University of Alberta

NEUROMODULATION OF RESPIRATORY CONTROL AND POTENTIAL PHARMACOTHERAPY TO ALLEVIATE RESPIRATORY DEPRESSION

by

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Abstract

The focus of this thesis is toward understanding the roles played by important neurochemical modulators on respiratory rhythm in physiological and pathophysiological conditions using *in vitro* and *in vivo* rodent models and developing a potential drug therapy to alleviate central respiratory depression. Three major studies were undertaken. 1) I determined the actions of $GABA_A$ and glycine receptor-mediated chloride conductances on respiratory rhythmogenesis in perinatal rats. That systematic study demonstrates that the transition from excitatory to inhibitory effects of chloride-mediated conductances on respiratory rhythmogenesis occurs at embryonic day 19 in the rat, regulated by the development of chloride co-transporters. I then demonstrated that $GABA_A$ receptor-mediated modulation of perinatal respiration is further influenced by neurosteroids. Collectively, those data demonstrate that the modulation of breathing by chloride-mediated conductances via glycine and $GABA_A$ receptor activation is determined by the developmental stage and overall balance of negative and positive neurosteroid modulators within respiratory nuclei. 2) I studied central respiratory disorders in a genetic mouse model. I tested *necdin*-deficient mice, one of a cluster of genes deleted in the neurodevelopmental disorder Prader-Willi syndrome (PWS). Both animal models and children with PWS have apparent respiratory insufficiency during the neonatal period. I demonstrate that the respiratory defect can be explained by abnormal neuronal activities within the putative respiratory rhythm-generating centre. Specifically,

the rhythm is unstable with prolonged periods of depression of respiratory rhythmogenesis. Exogenous application of excitatory neuromodulators alleviates slow respiratory rhythms, but some instability of rhythmogenesis persists. These observations may reflect abnormalities of respiratory rhythm-generating neurons and conditioning neuromodulatory drive. 3) My final research project was directed toward developing a novel and clinically relevant pharmacological means of alleviating respiratory depression. I demonstrate that the ampakine class of drugs (by modulating AMPA-type glutamate receptor) counters respiratory depression associated with opioid analgesics, anesthetics and weak endogenous drive. Importantly, the ampakine CX717, which is metabolically stable and safe for human use, can alleviate life-threatening opioid-induced respiratory depression without interfering with analgesia. Thus, CX717 could potentially improve the safety margin for administering powerful analgesic agents, which would provide a valuable tool for clinicians to optimize pain management in patients.

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List of symbols, nomenclature abbreviations

5-HT	5-hydroxytriptamine, serotonin
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Bdnf	brain-derived neurotrophic factor
Ca^{2+}	calcium ion
CCHS	congenital central hypoventilation syndrome
Cd^{2+}	cadmium ion
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CO_2	carbon dioxide
CPAP	continuous positive airway pressure
CREB1	cAMP-responsive element binding protein 1
CSA	central sleep apnea
DMSO	dimethylsulfoxide
DRG	dorsal respiratory group
E	embroynic
EMG	electromyography
EGTA	ethylene glycol-bis(b-aminoethylether)-N,N,N',N'-tetraacetic acid
FBM	fetal breathing movements
FFA	flufenamic acid
GABA	α-aminobutyric acid
GH	growth hormone
I_{CAN}	Ca^{2+}-activated non-specific cation current
I_{NaP}	persistent sodium current
I.P.	intraperitoneal
I.V.	intravenous
IR-DIC	infrared-differential interference contrast
MeCP2	methyl-CpG-binding protein 2 gene
MeCP2	methyl-CpG-binding protein 2
MECP2	human methyl-CpG-binding protein 2 gene
NA	nucleus ambiguous
Ndn	*necdin*
REM	rapid eye movement

NK1R	neurokinin 1 receptor
NTS	nucleus tractus solitarius
O_2	oxygen
OSA	obstructive sleep apnea
P	postnatal
PAP	positive airway pressure
pFRG	parafacial respiratory group
Phox2b	homeodomain transcription factor *Phox2b* gene
Phox2b	homeodomain transcription factor *Phox2b* gene product
PHOX2B	human homeodomain transcription factor *Phox2b* gene
preBötC	pre-Bötzinger complex
PRG	potine respiratory group
RTT	Rett syndrome
PWS	Prader-Willi syndrome
SA	sleep apnea
SIDS	sudden infant death syndrome
SP	substance P
SP-SAP	substance P conjugated to saporin
TRH	thyrotropin-relasing hormone
TTX	tetrodotoxin
VRC	ventral respiratory column
VRG	ventrolateral respiratory group

CHAPTER I

GENERAL INTRODUCTION

1.1 OVERVIEW

Breathing movements responsible for O_2 uptake and CO_2 removal are essential for survival. This occurs in mammals by ventilation of the lungs as a consequence of rhythmic contractions of respiratory muscles including the rib cage muscles and diaphragm innervated by spinal motoneurons. The numerous respiratory muscles of the chest wall and the upper airway valve must rhythmically contract in a higly-coordinated manner to efficiently induce and control airflow. The two ventilatory phases of inhalation and exhalation are controlled by the three neural phases of inspiration, post-inspiration and active expiration (Richter, 1982; Bianchi et al., 1995; Ballanyi et al., 1999). The neural circuits responsible for breathing must be capable of generating robust rhythm-driving ventilation by birth and can adjust to homeostatic needs regarding developmental and physical changes. They must also coordinate and integrate with other movements such as swallowing and locomotion. Breathing is generated and controlled by a brainstem neuronal network (Richter, 1982; Bianchi et al., 1995; Feldman, 1995; Feldman et al., 2003; Wong-Riley & Liu, 2005; Peña & García, 2006). Deficiencies of respiratory control may be involved in several respiratory disorders such as prematurity-, sleep-, and drug-related apnea. In addition, devastating disorders such as Prader-Willi Syndrome, Rett Syndrome cause significant respiratory instability. Understanding the pathogenesis of breathing problems in these maladies and development of effective pharmacological treatment will greatly benefit from knowledge of the central neural control of respiratory rhythm. Three critical aspects of respiratory control are of particular interest as they relate to my thesis.

Respiratory rhythm: Where, how and when is the respiratory rhythm generated and modulated? Understanding of these issues will provide us with insights of pathogenesis and pharmacotherapy of the central respiratory disorders.

Central respiratory disorders: Although several respiratory disorders are suspected to be caused by dysfunction in respiratory control, the underlying cellular and molecular mechanisms remain unclear. Genetic mouse models and other animal models may provide unique insights and opportunities for analysis of these devastating human respiratory disorders.

Management of disordered respiratory control: There is an emergent need for improved therapeutic interventions to treat various respiratory dysfunctions. Although several drugs are currently used clinically to treat central respiratory dysfunctions, the mechanisms involved in their effects on the respiratory control are poorly understood and improvements are desired.

1.2 RESPIRATORY RHYTHM

Respiratory neurons, defined as those that fire their action potentials related to the respiratory cycle include pre-inspiratory, inspiratory, post-inspiratory and expiratory neurons (Rekling & Feldman, 1998; Ballanyi et al., 1999). The respiratory nervous outflow in motoneurons supplying respiratory muscles originates from respiratory neurons in three major parts of the brainstem including the pons (pontine respiratory group, PRG), dorsal medulla (dorsal respiratory group, DRG) and ventral medulla (ventral respiratory column, VRC) (Fig. 1.1.) (Bianchi et al., 1995; Ballanyi et al., 1999; Richter & Spyer, 2001; Feldman et al., 2003; Wong-Riley & Liu, 2005). The respiratory nuclei localized in the pons include the nucleus parabrachialis and Köllicker–Fuse nucleus; also referred to as the pneumotaxic center. These propriobulbar neurons, while not essential for respiratory rhythmogenesis, modulate the rhythm and regulate airway muscles during exercise and sleep (Bianchi et al., 1995; Alheid et al., 2004; Richter & Spyer, 2001). Similarly, DRG neurons are not essential for respiratory rhythm generation but play important roles in modulating the respiratory rhythm. For example, the ventrolateral nucleus tractus solitarius (NTS) contains inspiratory neurons closely related to the processing of feedback information (Bianchi et al., 1995; Hilaire & Duron, 1999; Richter & Spyer, 2001; Feldman et al., 2003; Kubin et al., 2006). The preBötzinger Complex (preBötC), Bötzinger Complex (BötC) and retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) are rostral parts of a continuous VRC. Caudally within this column, excitatory inspiratory and expiratory premotor neurons are concentrated in regions referred to as the rostral (rVRG) and caudal (cVRG) ventral respiratory groups, respectively (Shen & Duffin, 2002; Wong-Riley & Liu, 2005). The BötC contains cranial motoneurons and a major group of inhibitory expiratory neurons (Feldman, 1986). The preBötC is located ventral to the semi-compact division of the nucleus ambiguous (NA), caudal to the compact division of the NA and rostral to the anterior tip of the lateral reticular formation (Fig. 1.1., Smith et al., 1991; Wong-Riley & Liu, 2005; Feldman & Del Negro, 2006). It contains respiratory neurons that are necessary for inspiratory rhythm generation (as outlined below). The pFRG contains primarily preinspiratory neurons and may function as a center for expiratory rhythm generation (Feldman & Del-Negro, 2006, see below). A major component of current studies in the field of respiratory control is directed toward understanding how neurons are organized to generate rhythmic drive.

1.2.1 RESPIRATORY RHYTHM GENERATION CENTERS

It has been hypothesized that there are two distinct respiratory rhythm generators in the medulla; the preBötC and pFRG. The hypothesis that the preBötC is the primary rhythm generator is supported by several observations. First, sequential serial sectioning from the brainstem–spinal cord neonatal rat preparation revealed that a restricted area of the medulla containing the preBötC was necessary and sufficient for generating respiratory rhythm *in vitro* (Smith et al. 1991; Rekling & Feldman, 1998; Feldman et al. 2003). Secondly, study of molecular markers explored a critical population of preBötC neurones with expression of neurokinin-1 receptors (NK1R; Gray et al. 1999). Subsequent studies supported the idea that there is a population of small fusiform, glutamatergic neurones in the preBötC expressing NK1R that have characteristics consistent with their involvement in rhythmogenesis (Pilowsky & Feldman, 2001; Wang et al., 2001). Thirdly, in adult rats, destruction of NK1R-expressing neurones within the preBötC by substance P conjugated to saporin results in dysfunctional breathing in a fixed sequence over a period of days. Sustained and repeated apneas first appear during rapid eye movement (REM) sleep, while breathing remains normal during wakefulness and non-REM sleep. These disturbances then spill over into non-REM sleep without any marked changes in breathing during wakefulness. Finally, ataxic breathing develops that extends into wakefulness (Gray et al., 2001; McKay et al., 2005, 2008). Fourthly, using adeno-associated virus 2, Tan et al. (2008) expressed the Drosophila allatostatin (non-endogenous) receptor in somatostatin-expressing neurons in the preBötC. They reported that rapid silencing of these neurons in awake rats induced a persistent apnea without any respiratory movements.

The notion that there is a second respiratory rhythm generating center was supported by recent studies using a combination of imaging, electrophysiological and pharmacological techniques, suggesting that neurones within pFRG are intrinsically rhythmogenic and may control expiratory musculature (Janczewski et al., 2002; Onimaru & Homma, 2003, 2005; Onimaru et al., 2006). How the preBötC cooperates with pFRG is currently under active investigation (Janczewski et al., 2002; Onimaru & Homma, 2003; Mellen et al., 2003). Under normal conditions in mammals, these two oscillators are well coordinated but may be dominated by the preBötC (Feldman & Del-Negro, 2006).

1.2.2 MECHANISMS OF RESPIRATORY RHYTHMOGENESIS

Although a central role of the preBötC for inspiratory rhythmogenesis in perinates and adults is widely accepted, there remains considerable debate about the cellular mechanisms (Richter & Spyer, 2001; Feldman et al., 2003; Ramirez et al., 2004; Del Negro et al., 2005; Feldman & Del Negro, 2006; Del Negro & Hayes, 2008). Initially, respiratory rhythm generation was explained by considering two models: (1) a network model, in which inhibitory synaptic interactions are essential for rhythmogenesis and (2) a pacemaker model, in which neurons with pacemaker properties underlie rhythmicity. The observation of persistence of respiratory rhythm *in vitro* after blockade of Cl^--mediated synaptic inhibition (Feldman & Smith, 1989), and the finding of neurons with pacemaker properties (Smith et al., 1991) involving persistent sodium or calcium-dependent non-specific cationic currents (Thoby-Brisson & Ramirez, 2000) supported the pacemaker hypothesis. In one model, both types of pacemaker neurons are necessary for eupneic activity in the medullary slice preparation. This is based on the fact that co-application of riluzole, a blocker of persistent sodium currents (I_{NaP}) and flufenamic acid (FFA), a blocker of Ca^{2+}-activated non-specific cation currents (I_{CAN}) silences the respiratory rhythm in rodent slices *in vitro* (Peña et al., 2004; Del Negro et al., 2002a, 2005). However, the results must be interpreted with caution due to unspecific nature of riluzole and FFA. For example, in addition to blocking I_{NaP}, riluzole depresses excitatory transmission (Doble, 1996; Wang et al., 2004), and causes the XII motor discharge to decline in amplitude (Del Negro et al., 2005). Further, almost all preBötC neurons and hypoglossal motoneurons express I_{NaP} (Del Negro et al., 2002b; Rybak et al., 2003); and I_{NaP} was significantly enhanced with elevated extracellular concentrations of potassium. These drugs also lower overall neuronal excitability throughout the network: blocking I_{NaP} hyperpolarizes baseline membrane potentials and blocking both currents removes inward currents that ordinarily enhance inspiratory synaptic drive. Therefore, the loss of rhythm could simply be due to riluzole and FFA lowering the excitability of neurons, regardless of their effects on pacemaker properties. Interestingly, local microinjection in the preBötC of riluzole does not perturb respiratory frequency, even in the presence of bath-applied FFA. However, in the presence of FFA, local microinjection of riluzole in the raphe obscurus causes rhythm cessation, suggesting that I_{NaP} regulates the excitability of neurons outside the preBötC including serotonergic raphe neurons that project to and help maintain rhythmic preBötC function. Recently, contradictory data from *in situ* brainstem-working heart preparations suggests that the respiratory rhythm generation during gasping but not eupnea depends on the persistent sodium currents (Paton et al., 2006). An alternate emergent network-based mechanism referred to as the group-pacemaker hypothesis has been proposed in which ensembles of neurons become rhythmically active through chemical and excitatory synaptic interactions (Rekling & Feldman, 1998; Feldman & Del Negro, 2006; Del Negro & Hayes, 2008). Overall, this important matter regarding cellular mechanisms underlying respiratory rhythmogenesis has not been adequately resolved.

1.2.3 NEUROCHEMICAL MODULATION OF RESPIRATORY RHYTHM

A primary goal of respiratory research is to identify the neurotransmitter systems responsible for modulating respiratory rhythmogenesis and motoneuron drive. An understanding of the neurochemical control of breathing prenatally should provide insights into the mechanisms underlying episodic fetal breathing movements (FBM) that are important for the maturation of lungs and respiratory neuromuscular systems (Kitterman, 1988; Harding & Hooper, 1996; Greer et al., 1999). The occurrences of hypoxia, drug, sleep-induced apnea in newborns and adults are related to altered levels of neurochemical modulators within medullary respiratory nuclei (Jansen & Chernick, 1991; Bonham, 1995; Ballanyi, 2004).

The primary excitatory drive that maintains the oscillatory state in the preBötC arises from activation of non-NMDA glutamatergic receptors (primarily AMPA receptors; Greer et al., 1991; Funk et al., 1993; Thoby-Brisson et al., 2005). Blockade of non-NMDA receptors with the antagonist CNQX causes a dose-dependent decline, and eventual cessation, of respiratory frequency and inspiratory drive to cranial and spinal motoneurons. Elevation of endogenously released glutamate levels with glutamatergic uptake inhibitors or reduction of AMPA receptor desensitization (Funk et al., 1995) leads to increases in respiratory frequency *in vitro*. Further conditioning is provided by a diverse group of neuromodulators (Ballanyi, 2004; Peña & García, 2006), including those having excitatory actions on respiratory rhythmogenesis such as substance P (SP) and thyrotropin releasing hormone (TRH) and inhibitory neuromodulators such as opioids, prostaglandins, γ-aminobutyric acid (GABA), glycine, somatostatin, and adenosine and neuromodulators with mixed effects such as serotonin (5-HT), ATP, and noradrenaline (NA). SP, acting on the NK1 receptor, depolarizes respiratory neurons present in the preBötC, by activating a TTX insensitive Na^+ current and by increasing pacemaker activity (Peña et al., 2004). NA either increases or decreases respiratory rhythm with species dependency primarily attributed to the relative expression of $\alpha 2$ and $\alpha 1$ receptors (Hilaire & Duron, 1999). 5-HT is another neuromodulator with a complex effect on respiratory rhythm generation due to the differential effects of various 5-HT-receptor subtypes

(Ballanyi, 2004). There is evidence that 5-HT$_{2A}$ receptor activation is required for proper pacemaker function (Ramirez et al., 2004) and that this receptor may be critical to the generation of fictive eupnea during normoxia (Peña & Ramirez, 2002) and fictive gasping activity during hypoxia *in vitro* (Tryba et al., 2006), by modulating the activity of both persistent and transient Na$^+$ currents (Ramirez et al., 2004). However, contradictory data from a recent study suggested that 5-HT is not necessary for either eupnea or gasping *in vivo* (Toppin et al., 2007). Opioids, commonly used and effective analgesics, have a significant inhibitory effect on respiratory rhythm, by acting primarily on μ-receptors, opening a K$^+$ conductance and hyperpolarizing respiratory neurons both *in vivo* and *in vitro* (Greer et al., 1995; Ballanyi et al., 1997; 2004; Dahan et al., 2001; Lalley, 2003; McCrimmon & Alheid, 2003; Pattinson, 2008).

1.2.4 DEVELOPMENT OF CENTRAL RESPIRATORY CONTROL

The NK1R expression data provided the basis for a study of the ontogeny of the preBötC in the rat (Pagliardini et al., 2003). The NK1R-expressing neurones are born ~E13, and reach the region of the preBötC by ~ E17 (Fig. 1.2, Greer et al., 2006). This coincides with the time that respiratory-related neural discharge is first detected electrophysiologically *in vitro* and via ultrasound recordings *in vivo* (Kobayashi et al., 2001). Fitting with the two days shorter gestation period of the mouse, the onset of inspiratory discharge *in utero* and *in vitro* mouse preparation is E15 (Viemari et al., 2003; Thoby-Brisson et al., 2005). Less is known about the prenatal development of the pFRG. However, imaging of respiratory network activity in rat with voltage-sensitive dyes suggests that rhythmic activity of the pFRG commences at E18, ~ 1 day later than in the rat preBötC (Onimaru & Homma, 2005). By birth, the regulatory neural network responsible for respiratory control is capable of generating robust rhythm-driving ventilation that can adjust to homeostatic needs (Greer et al., 2006). The medullary respiratory network of mammals is more mature at birth compared with many other neural systems, although there are significant changes in morphology, synaptic control and membrane properties of respiratory neurons with maturation continuing for several postnatal weeks (Richter & Spyer, 2001).

An important developmental aspect for neural control of respiratory rhythm is the evidence of a dramatic decrease in tolerance of the respiratory network of rodents to metabolic disturbances within ~ ten days after birth. The developmental change is primarily due to a decrease in the ability of respiratory neurons to utilize anaerobic metabolism (Ballanyi et al., 1999). A second major developmental difference is the level of electrical couplings (gap junctions) among respiratory neurons. At the onset of respiratory rhythmogenesis, extensive gap junction connections are proposed to be responsible for the widespread non-respiratory rhythm (Ren & Greer, 2003; Thoby-Brisson et al., 2005). Functional gap junctions persist during the first two postnatal weeks (Rekling et al., 2000). This produces a strong and instantaneous synchronization of neonatal neurons that differs significantly from the gradual summation of postsynaptic activity in mature neurons.

Neuronal input resistance and excitability decline with both the postnatal extension of the dendritic tree and the increase in the number of synapses (Hilaire & Duron, 1999), which are formed as axonal projections within and beyond the network (Fitzgerald & Jennings, 1999). Incomplete connectivity, structural and functional immaturity of synaptic interactions and signal integrations in medullary neurons appear to be responsible for (1) the long delays between central respiratory and output activities (Richter & Spyer, 2001), and (2) slow rhythm (Greer et al., 2006) in prenatal and neonatal animals. On the other hand, the increase in respiratory frequency and stability over development reflects (1) the maturation of respiratory neurons and network underlying rhythmogenesis, (2) decreased suppression of network activity by endogenous inhibitory modulators, or (3) enhanced modulatory systems that provide excitatory drive to the respiratory networks. Further, exogenous application of excitatory neuromodulators such as SP increases the respiratory frequency and stability, demonstrating the slow perinatal rhythm is not due to inherent limitations of rhythmic generating centers, but rather because of a lack of necessary excitatory conditioning drive from neuromodulators (Greer et al., 2006).

These neuromodulation studies provide a rationale of pharmacotherapy to stimulate weak and irregular central respiratory drive in perinates.

1.2.5 EXPERIMENTAL MODELS TO STUDY THE CENTRAL RESPIRATORY CONTROL

Rodent models for studying the neural control of respiratory rhythm include (1) *in vivo* plethysmographic recordings, (2) perinatal *in vitro* brainstem-spinal cord and medullary slice preparations, and (3) *in situ* working heart-brainstem preparation. It is essential to compare the various experimental approaches and to address the differences among experimental data (Richter & Spyer, 2001; Peña & García, 2006). The *in vivo* approach is the best way for the study of the behaviour, since neural control of breathing is performed under the intact influence of complex afferent information coming from the periphery and the rest of the brain, which is eliminated in more reduced preparations (Ballanyi et al., 1999). However, the obvious disadvantage of an *in vivo* approach is that intracellular recordings are difficult to perform under the condition of ongoing modulation by a multiplicity of excitatory and inhibitory afferent inputs. Therefore, it is difficult to explore the cellular mechanisms involved in the neuronal control of breathing from *in vivo* experiments. *In vitro* preparations have been developed to overcome the *in vivo* limitation, including en bloc brainstem–spinal cord (Suzue, 1984) and medullary slice (Smith et al., 1991) preparation from rodents. Brainstem-spinal cord preparation contains two putative respiratory rhythmogenesis centers (preBötC and pFRG) and is capable of generating respiratory rhythm including inspiratory and expiratory bursts. Medullary slice preparation contains only the preBötC and necessary synaptic interactions. The slice preparation produces stable fictive inspiratory rhythm in the presence of elevated concentration of extracellular potassium in order to overcome the loss of most excitatory inputs preserved in the brainstem-spinal cord preparation. The development of *in vitro* models has significantly advanced our understanding of mechanisms underlying the respiratory control. The seminal study that generated a rhythmic medullary slice by sequential sectioning of the en bloc preparation revealed that the preBötC was necessary and sufficient for inspiratory rhythm generation (Smith et al., 1991). Recently, Ruangkittisakul et al. (2006) have reported that a stable inspiratory rhythm could be generated in the on-line-calibrated newborn rat medullary slice preparation in the physiological (3 mM $[K^+]_o$) solution. The use of *in vitro* preparations has allowed the application of more sophisticated pharmacological, electrophysiological and imaging approaches, under more controlled conditions, providing a deeper understanding of respiratory rhythm generation and control (Smith et al., 1991; Koshiya & Smith, 1999). However, *in vitro* preparations are only viable in fetal and early postnatal periods, in part due to difficulty of diffusion of glucose and O_2 into core of preparations from the older animals. For this reason, a transition between *in vitro* and *in vivo* approaches, the *in situ* working heart-brainstem perfused preparation has been developed. The advantages of this preparation include 1) the tissue is well oxygenated and 2) the experimental conditions allow for the use of extracellular and intracellular techniques (Richerson & Getting, 1990; Paton, 1996; Paton & St-John, 2005; Bradley et al., 2008).

It is noted the relevance of *in vitro* preparations was questioned by some research groups based on the fact that they do not generate the same augmenting inspiratory and declining post-inspiratory activity pattern seen under *in vivo* conditions (Bianchi et al., 1995) or in the *in situ* perfused brainstem of mature rodents (Paton, 1996). However, the difference of this rhythm observed with *in vitro* preparations from eupnic breathing of in intact neonatal rats is, in part, secondary to removal of afferent inputs in the course of the isolation of the medulla and to the decreased *in vitro* temperature (Ballanyi et al., 1999). Furthermore, a clear distinction among qualities of the various preparations using the criterion of the presence of post-inspiratory activity is questionable (Dutschmann et al., 2000; Richter & Spyer, 2001). The declining rather than augmenting pattern of inspiratory activity of *in vitro* neonatal preparations might disguise the existence of post-inspiratory components of activity. Overall, the various preparations define different operational conditions of the network, including maturation of neurons and synaptic processes (Richter & Spyer, 2001).

1.3 CENTRAL RESPIRATORY DISORDERS

Lung and cardiac pathologies are the main causes of breathing disorders, but dysfunctions relating to the neural control of breathing also have a significant impact on public health. Such dysfunctions include apnea of prematurity, sleep apnea, and possibly sudden infant death syndrome (SIDS). Abnormal respiration is also observed in several genetic disorders including congenital central hypoventilation syndrome, Prader-Willi syndrome, and Rett syndrome. The respiratory depression caused by drug abuse, opioids or anaesthetics and spinal cord injury presents an emergent challenge on public health. Death due to central respiratory arrest is common in neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease and multiple systems atrophy (Feldman & Del Negro, 2006). In the following, I will expand upon some of these potentially devastating respiration dysfunctions.

1.3.1 SLEEP APNEA (SA)

Apnea is defined as the cessation of breathing. SA is a sleep disorder characterized by pauses in breathing during sleep, defined as the interruption of breathing for at least 10 seconds and at least five times per hour during sleep. During SA, the induced hypoxia often results in exceptionally high blood pressure that can induce fatal cardiovascular or cerebrovascular incidents (Leung & Bradley, 2001; Pack, 2006). SA may also result in secondary depression, insomnia, memory problems, weight gain, impotency and an increased incidence of motor vehicle crashes (Young et al., 2002; Pack, 2006). SA has been classified as obstructive sleep apnea (OSA), central sleep apnea (CSA), and mixed apnea (a combination of OSA and CSA).

OSA is the most common of apneas, caused by blockage of the airway, usually when the soft tissue in the rear of the throat collapses and closes during sleep. Although the primary cause of OSA remains uncertain, abnormalities of airway structure, such as enlarged tonsils and uvula, and abnormal airway function may contribute to the OSA. In contrast to OSA in which ongoing respiratory efforts are observed, CSA is defined by a lack of respiratory effort during cessations of airflow. The mechanisms underlying CSA are unclear, although it is proposed to be caused by a dysfunction of the centers that generate and control breathing. Breathing is more irregular and spontaneous central apneas are most frequent during REM sleep in both rats (Mendelson et al., 1988) and humans (particularly the elderly, Krieger et al., 1983a). The pathophysiology of SA namely, normal respiration in waking with disordered breathing only during sleep, implicates the involvement of the state-dependent neurochemical mechanisms in respiratory control. Neurons that release 5-HT or NA are relatively inactive during sleep, especially in REM (Siegel, 2004). As 5-HT and NA excite preBötC neurons, any decrease in their release would disfacilitate preBötC neurons, rendering breathing during REM vulnerable to preBötC depression. This hypothesis is supported by experiments of SP-SAP−induced lesions in preBötC (Gray et al. 2001; McKay et al., 2005; McKay & Feldman, 2008). As preBötC dysfunction progresses, disturbances in breathing would be expected initially in REM, next in non-REM, and, if sufficiently severe, even during wakefulness. Substantial damage to the ventrolateral medulla (including the presumptive preBötC) has also been proposed to be responsible for SA commonly seen in the later stage of neurodegenerative diseases such as multiple systems atrophy (MSA) or Parkinson's disease. This hypothesis is based on the findings of a decrease of 60% NK1R neurons in individuals with Parkinson's disease and a decrease of 89% NK1R neurons in individuals with MSA in the ventrolateral medulla (including the presumptive preBötC) (Benarroch et al., 2003). On the other hand, age-related defects in the serotonergic nervous system, associated with SA, could underlie deficits in upper airway tone during sleep (Nakano et al., 2001; Feldman et al., 2003).

1.3.2 APNEA OF PREMATURITY

Apnea of prematurity has been defined most widely as cessation of breathing longer than 20 s duration, typically accompanied by a decrease in PaO_2, arterial oxygen desaturation and bradycardia in preterm infants. Similar to SA, apnea of prematurity is classified traditionally into three categories based on the presence or absence of upper airway obstruction: central, obstructive, and mixed. In premature infants, inadequate prenatal maturation of the respiratory apparatus typically results from deficiencies of central respiratory rhythmogenesis or activation of respiratory musculature (Martin & Abu-Shaweesh, 2005).

The hypoxic ventilatory response has been well characterized in preterm infants (Rigatto, 1975; Gauda et al., 2004). During exposure to hypoxia, neonates exhibit a biphasic ventilatory response that consists of an initial increase in ventilation that lasts for 1–2 min, followed by a decline in breathing, often to below baseline ventilation. The mechanisms underlying the initial augmentation of central respiratory activity during hypoxia have been contributed to an activation of arterial chemoreceptors (Gauda et al., 2004). ATP-gated ion channels via P2X2 receptors may play an important role in the peripheral (Rong et al., 2003) and central (Lorier et al., 2008) ventilatory stimulating response to hypoxia. The processes responsible for hypoxic ventilatory depression and hypoxic apnea are less well understood. Depression of neuronal excitability and synaptic interactions between medullary respiratory neurones (Richter et al., 1991) and increased release of inhibitory neuromodulators (e.g. GABA, adenosine, endorphins) were proposed to mediate the late hypoxic–induced respiratory depression (Melton et al., 1990; Neubauer et al., 1990; Richter et al., 1999; Martin et al., 2004; Martin & Abu-Shaweesh, 2005). However, there were inconsistent and contradictory observations. For example, Kato et al. (2000) reported that hypoxic-induced depression depends on glycinergic and opioid-mediated neuronal inhibition, but not GABA or adenosine systems in the rat *in vitro* preparation. Although increased release of adenosine was observed in NTS during the hypoxic challenge, it did not appear to mediate hypoxic-induced respiratory depression in rats (Gourine et al., 2002). A recent study (Hehre et al., 2008) suggested that the larger depression in the ventilatory response to hypoxia observed in younger piglets is mediated by predominance of the inhibitory neurotransmitters including GABA, glycine, and taurine, in the NTS. Furthermore, the depressive response to hypoxia is diminished by experimental lesions in the upper brainstem and midbrain of fetal lambs, implicating the presence of descending inhibitory tracts that contribute to hypoxic ventilatory depression (Gluckman &

Johnston, 1987). While it is unclear whether hypoxic ventilatory depression plays a role in initiating apneic events, once hypoxia occurs it may aggravate apnea and result in delayed recovery.

1.3.3 SUDDEN INFANT DEATH SYNDROME (SIDS)

SIDS is defined as the sudden death of an infant less than 1 year of age that remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene and review of the clinical history (Willinger et al., 1991). SIDS is the major cause of death in infants from 1 month to 1 year of age in developed countries. Significant progress in reducing the rate of SIDS has been accomplished by the move toward prone sleeping position for infants (in conjunction with avoidance of cigarette smoke exposure and overheating of the infant). The finding of an intervention that decreased SIDS rates helped remove some, but not all, of the mystery surrounding these unexplained deaths.

Although dysfunction of respiratory control was most widely assumed as the primary cause of SIDS, no clinical evidence reliably links a ventilatory control abnormality to SIDS. The apparent lack of a relationship between persistent apnea of prematurity and SIDS has become clearer in recent years. Although clinical case reports indicate deficiency of muscarinic, serotonergic and kainate receptor binding in the arcuate nucleus of the ventral medullary surface or diminished tyrosine hydroxylase immunoreactivity in the vagal nuclei and reticularis superficialis ventrolateralis in the SIDS brain (Kinney et al., 1995), these defects are not found in most SIDS victims. It has recently been shown that mutations in the *Krox-20* gene (a homeobox gene important for hindbrain morphogenesis and required for normal development of the central pattern respiratory generator; see Schneider-Maunoury et al., 1993) produces an abnormally slow respiratory rhythm and increased incidence of respiratory pauses. Inactivation of *Krox-20* may result in the absence of a rhythm promoting reticular neuron group localized in the caudal pons and could thus be a cause of life-threatening apnea during early infancy (Jacquin et al., 1996). This finding may be related to the apnea and bradycardia prevalent in SIDS victims. The failure to generate gasping and autoresuscitation assumed to be a major component of SIDS pathology. SP-SAP–induced preBötC NK1R neuron ablation in rats significantly reduces the inspiratory frequency and blunts regulatory responses to hypoxia and hypercapnia (Gray et al., 2001; McKay et al., 2005; McKay & Feldman, 2008); in humans, this could further increase the arousal threshold (Berry & Gleeson, 1997). Ultimately, a sleep apnea not terminated by arousal could result in anoxia and death. Interestingly, mice deficient in a transcription factor necessary for the production of most serotonergic neurons were found to have no significant alterations in breathing pattern in normoxia. This is surprising given the hypothesized critical role of serotonin for respiratory control and proposed link to SIDS (Gray, 2008). Overall, the cause and pathogenesis of SIDS remain unclear.

1.3.4 CONGENITAL CENTRAL HYPOVENTILATION SYNDROME (CCHS)

CCHS is a rare neurological disorder, in which the automatic control of breathing is impaired during asleep, but the voluntary control of ventilation which operates in awake hours is generally intact (Weese-Mayer et al., 1999). Central chemoreception is absent or reduced in children with CCHS (Feldman et al., 2003). These children function relatively normally during wakefulness but require ventilatory support during sleep to avoid very high CO_2 and low O_2 levels caused by inadequate breathing. The etiology is unknown, but some studies suggest that the *Rnx*, *Phox2b* (a paired-like homeobox gene), *Hash-1* and *Nurr1* genes are critical for the development of the ventral medullary respiratory center and its deficiency could be associated to CCHS (Amiel et al., 2003). The gene for CCHS has recently been further identified as *PHOX2B*, located on chromosome 4p12 (Berry-Kravis et al., 2006). Most cases of CCHS are heterozygous for the *PHOX2B* polyalanine repeat expansion mutation, with alternative mutations in *PHOX2B* in the other cases. Although most of the non-polyalanine expansion mutations occur de novo, some are inherited from otherwise asymptomatic carriers, suggesting that genetic screening of parents is important once such a mutation has been identified in a child. In addition, currently asymptomatic parents or siblings who carry this mutation may also be at some risk for subsequent development of nocturnal hypoventilation or late-onset atypical CCHS (Trochet et al., 2008). The hypothesis that, although CCHS is typically identified in newborns, it can also present in adults, was supported with *PHOX2B* mutation-confirmed CCHS (Antic et al., 2006; Trochet et al., 2008). In two CCHS patients diagnosed by identification of the *PHOX2B* polyalanine expansion mutation, magnetic resonance imaging also showed evidence of pontine hypoplasia and a Chiari I malformation, respectively (Bachetti et al., 2005). Since the guidelines from the American Thoracic Society state that CCHS is diagnosed in the absence of an identifiable brainstem lesion (Weese-Mayer et al., 1999), this criterion may be too restrictive, and strict adherence to these guidelines led to the initial exclusion of a diagnosis of CCHS. Recently, Dubreuil et al. (2008) reported that *Phox2b*-mutant mice breathe irregularly, do not respond to an increase in CO_2, and die soon after birth from central apnea. The *Phox2b* mutants specifically lack Phox2b-expressing glutamatergic neurons located in the parafacial region, whereas other sites known or supposed to be involved in the respiratory control are anatomically normal.

1.3.5 PRADER-WILLI SYNDROME (PWS)

PWS is a developmental neurobehavioral disorder that occurs sporadically at a frequency of ~1 in 15,000 (Holm et al., 1993). PWS, originally described by Prader, Labhart, and Willi in 1956, is characterized by failure to thrive, early obesity starting during the second year of life, hypotonia, short stature, other endocrine dysfunctions, learning disabilities, abnormal behaviour, and psychiatric problems. Infants with PWS have significant respiratory abnormalities, including sleep-related central and obstructive apneas and reduced response to changes in oxygen and CO_2 levels (Arens et al., 1994; Clift et al., 1994). Four known protein-coding genes that are deficient in people with PWS, including the gene encoding Ndn, are active only on the paternally inherited allele and silenced by imprinting on the maternal allele (Nicholls, 2000). The relative contribution of the loss of each gene to the complex PWS phenotype is unknown, and there are no known cases of PWS attributable to deficiency of only one protein-encoding gene. Two of three *necdin*-deficient mouse strains generated demonstrated neonatal lethality of variable penetrance due to severe hypoventilation (Tsai et al., 1994; Gerard et al., 1999; Muscatelli et al., 2000). However, the mechanism underlying the respiratory dysfunction was unclear, which is part of my study undertaken in this thesis project.

Growth hormone (GH) treatment has been reported to significantly modify the life of children with PWS (Burman et al., 2001), not only by accelerating growth velocity but by also decreasing fat mass while increasing muscle mass, strength, and respiratory capacity. However, a review (Tauber et al., 2008) of 64 cases of death among individuals aged 20 years or less with PWS during the period 1980-1997 showed that respiratory disorders were the most common cause of death (respiratory insufficiency or infections), which were reported in 61% of the children (68% in GH-treated and 55.5% in -untreated patients). No significant differences in gender, prevalence of obesity or prevalence of sleep apnea were found between the patients treated with GH and the untreated patients. The fact that the first 9 months of GH treatment seems to be a high-risk period emphasizes the need for comprehensive care before and during GH treatment, or an alternative therapy.

1.3.6 RETT SYNDROME (RTT)

RTT is a complex neurological disorder first described in 1966 by Dr. Andreas Rett (Rett 1966). This severe neurodevelopmental disorder occurs almost exclusively in females, with an incidence of ~1:15,000. Girls with classical RTT show progressive loss of intellectual functioning, fine and gross motor skills and communicative abilities, deceleration of head growth, and the development of stereotypic hand movements at 6-18 months of age after apparently normal development. The discovery that RTT is caused by mutations of the methyl-CpG-binding protein 2 (MeCP2) provided a major breakthrough toward understanding this disorder (Amir et al., 1999). Animal models and expression studies have contributed to defining the role of MeCP2 in functional development (Chen et al., 2001; Guy et al., 2007). *In vitro* assays and microarray studies have delineated the potential molecular mechanisms of *MeCP2* functioning as the transcriptional silencing of specific target genes such as brain-derived neurotrophic factor expression (*Bdnf*, Chang et al., 2006; Zhou et al., 2006). Very recently, Chahrour et al. (2008) examined gene expression patterns in the hypothalamus of mice that either lack or overexpress MeCP2. In both models, MeCP2 dysfunction induced changes in the expression levels of thousands of genes, but unexpectedly the majority of genes (approximately 85%) appeared to be activated by MeCP2. Six genes were selected and confirmed that MeCP2 binds to their promoters. Furthermore, MeCP2 was associated with the transcriptional activator cAMP-responsive element-binding protein 1 (CREB1) at the promoter of an activated target but not a repressed target. These studies suggested that MeCP2 regulates the expression of a wide range of genes in the hypothalamus and that it can function as both an activator and a repressor of transcription.

Girls with RTT often develop significant breathing instability characterized by alternate hyperventilation and breath holding/apnea in wakefulness, with relatively normal breathing during sleep (Weese-Mayer et al., 2006). Recent studies of the *MeCP2*-deficient mouse suggested that the breathing dysfunctions are related to a lack of neuromodulators that play an important role in regulating respiratory rhythmogenesis within the brainstem (Viemari et al., 2005) or a deregulation of *Bdnf* (Wang et al., 2006). Further, the report that Bdnf expression and respiratory function modestly improve after ampakine treatment in a mouse model of RTT, suggested a potential pharmacological means of alleviating some features of this devastating syndrome (Ogier et al., 2007).

1.3.7 AMYOTROPIC LATERAL SCLEROSIS (ALS)

ALS, often referred to as "Lou Gehrig Disease", is a progressive neurodegenerative disorder of the voluntary motor system. People of all races and ethic backgrounds are affected with approximately 1 in 50,000 new cases of ALS each year. The disorder causes skeletal muscle weakness and atrophy throughout the body as motor neurons degenerate and die, ceasing to send messages to muscles. Eventually, the brain completely loses its ability to initiate and control voluntary movement, and leads to the development of respiratory failure, the usual cause of death within 3-5 years from onset of symptoms (Kaplan & Hollander., 1994).

ALS is primarily classified into two groups: 1) Sporadic ALS (SALS), constituting ~90% of all cases and having no known hereditary component. 2) Familial ALS (FALS) caused by genetic factors, accounting for remaining ~10% of all ALS cases. Although the cause of ALS is not known, ~10-20% of FALS is linked to a mutation in superoxide dismutase (*SOD1*), a copper/zinc dependant dismutase that is responsible for scavenging free radicals (Turner & Talbot, 2008). SALS is believed to be a multi-factorial disease in which modifying genes and environmental agents affect its clinical manifestation. Recently, genetic variations in two hypoxia-inducible angiogenic genes, the vascular endothelial growth factor (*VEGF*, see Lambrechts et al., 2003; Bogaert et al., 2006) and *angiogenin* (*ANG,* see Greenway et al., 2006; Wu et al., 2007; Gellera et al., 2008) have been linked with SALS. Data linking these two genes with SALS implicates a potential role for other similar hypoxia-responsible genes acting upon a common pathway that is crucial to motor neuron survival (Lambrechts et al., 2006). However, further screening of hypoxia-inducible genes in SALS patients failed to reveal association of common variation across 24 candidate hypoxia-inducible angiogenic genes with SALS (Cronin et al., 2008). Deletion of *VEGF* caused motor neuron disease phenotype in mice (Oosthuyse et al., 2001; Lambrechts et al., 2003), while *VEGF* delivery has shown promising neuroprotective properties in ALS rodent models (Azzouz et al., 2004; Storkebaum et al., 2005).

In ALS, neurons with low levels of Ca^{2+} buffers (Alexianu et al., 1994), including motoneurons and preBötC neurons, are particularly vulnerable to degeneration. If preBötC neurons and phrenic motoneurons were to degenerate, then significant disturbances of breathing would be expected, although this has not yet been confirmed. Molecular mechanisms such as glutamate-induced excitotoxicity, axonal transport blockade, mitochondrial dysfunction, neuroinflammation and apoptosis triggered by mutant *SOD1* catalysed oxidative reactions and/or protein misfolding are proposed to drive ALS pathogenesis (Rowland, 1991). One hypothesis holds that glutamate, the primary excitatory neurotransmitter in the CNS, accumulates to toxic concentrations at synapses and causes neurons to die, probably through a calcium-dependent pathway. Supporting this hypothesis are observations of abnormal glutamate metabolism (Plaitakis, 1991), and decreased high-affinity glutamate uptake by synaptosomes from the spinal cord and motor cortex (Rothstein et al., 1992). Drugs that modulate the glutamatergic system have been proposed as possible treatment in ALS. A controlled clinical trial demonstrated that the anti-glutamate release agent riluzole appeared to slow the progression of ALS, and it may improve survival in patients with disease of bulbar onset (Bensimon et al., 1994). However, results on the efficacy of riluzole (to date the only treatment) in observational population-based studies with a longer follow-up are conflicting and it is still unclear if the effect of the drug is limited to an early stage of the disease and to some specific subgroups of patients.

1.3.8 DRUG-INDUCED RESPIRATORY DEPRESSION

Several drugs commonly used in clinical medicine may cause significant respiratory depression. While it has been reported that only ~ 1% of total adverse drug events caused by prescription medications are respiratory in nature, these account for 25%-30% of drug-induced expected deaths. These events usually occur during the dose adjustment period or when different central nervous depressants are taken together without checking with a pharmacist or physician. Alcohol, opioids, and barbiturates are the primary classes of drugs responsible for these effects (Peña & Garcia, 2006). Heavy alcohol consumption may cause many neurological disorders including severe respiratory disturbances, primarily via several neurotransmitter receptors and ion channels (Brailowsky & Garcia, 1999). Opioids produce direct inhibition of respiratory neurons in the ventral respiratory group primarily mediated by the activation of μ-receptors (Ballanyl et al., 1997; Lalley, 2003; Pattinson, 2008). Clinicians are currently concerned that the only means of countering opioid-induced respiratory depression is to give an opioid receptor antagonist. This can lead to considerable problems in managing pain. It was reported that the same neurons that strongly express opioid receptors also have 5-HT$_{4A}$ receptors, and that an agonist of the latter prevents opioid-induced respiratory depression without affecting the analgesic properties of the opioids in rats (Manzke et al., 2003). However, a 5-HT_{4A} agonist was not effective in alleviating opioid-induced respiratory suppression in humans (Lotsch et al., 2005). In my thesis, advances toward this goal have been developed.

1.4 MANAGEMENT OF DISORDERED RESPIRATORY CONTROL

Despite great strides in our understanding of respiratory neurobiology, therapeutic strategies for disorders in respiratory control have changed little in the past 30 years. Positive airway pressure and methylxanthine therapy remain the mainstays of therapy while other therapeutic approaches have been inadequately studied.

1.4.1 POSITIVE AIRWAY PRESSURE (PAP)

PAP was first developed for the treatment of OSA (Sullivan et al., 1981). It is also used for critically ill patients with respiratory failure, and apnea of prematurity. In these patients, PAP ventilation can prevent the need for endotracheal intubation, or allow earlier extubation. Continuous Positive Airway Pressure (CPAP with fixed-pressure) machine minimizes OSA by delivering a stream of compressed air via a hose to a nasal pillow, nose mask or full-face mask, splinting the airway (keeping it open under air pressure) so that unobstructed breathing becomes possible, reducing and/or preventing apneas and hypopneas. This has the additional benefit of reducing or eliminating snoring. CPAP treatment can be highly effective for the treatment of OSA. For some patients, the improvement in the quality of sleep and quality of life due to CPAP treatment is noticed after a single night's use. However, dangerous hypoxaemia could occur during CPAP treatment of OSA (Krieger et al., 1983b). Commonly reported adverse effects of CPAP include irritation, pain, rash, or skin breakdown at mask contact points, particularly the bridge of the nose, or within the nares when nasal pillows are used. Dryness or irritation of the pharyngeal membranes, nasal congestion and rhinorrhea, and eye irritation from air leakage are also common. Prospective CPAP candidates are often reluctant to use this therapy, since the nose mask and hose to the machine look uncomfortable and clumsy, and the airflow required for some patients can be vigorous. Some patients adjust to the treatment within a few weeks, others struggle for longer periods, and some discontinue treatment entirely (Rolfe et al., 1991; Basner, 2007). For treating patients with CSA, an alternate technique called xPAP ST (Spontaneous Time) is used to force a number of set breaths per minute, however, the benefits have not been well documented. Additional modes of pressure delivery have been developed (e.g. bilevel PAP, autoadjusting PAP, flexible PAP) since CPAP treatment of OSA was described, however, none of the variants of PAP improves adherence in unselected patients compared to CPAP. Overall, despite the increase in PAP treatment options, lack of acceptance and inadequate adherence to PAP therapy remain the major causes of treatment failure (Kakkar & Berry, 2007).

1.4.2 PHARMACOTHERAPY IN CENTRAL RESPIRATORY DISORDERS

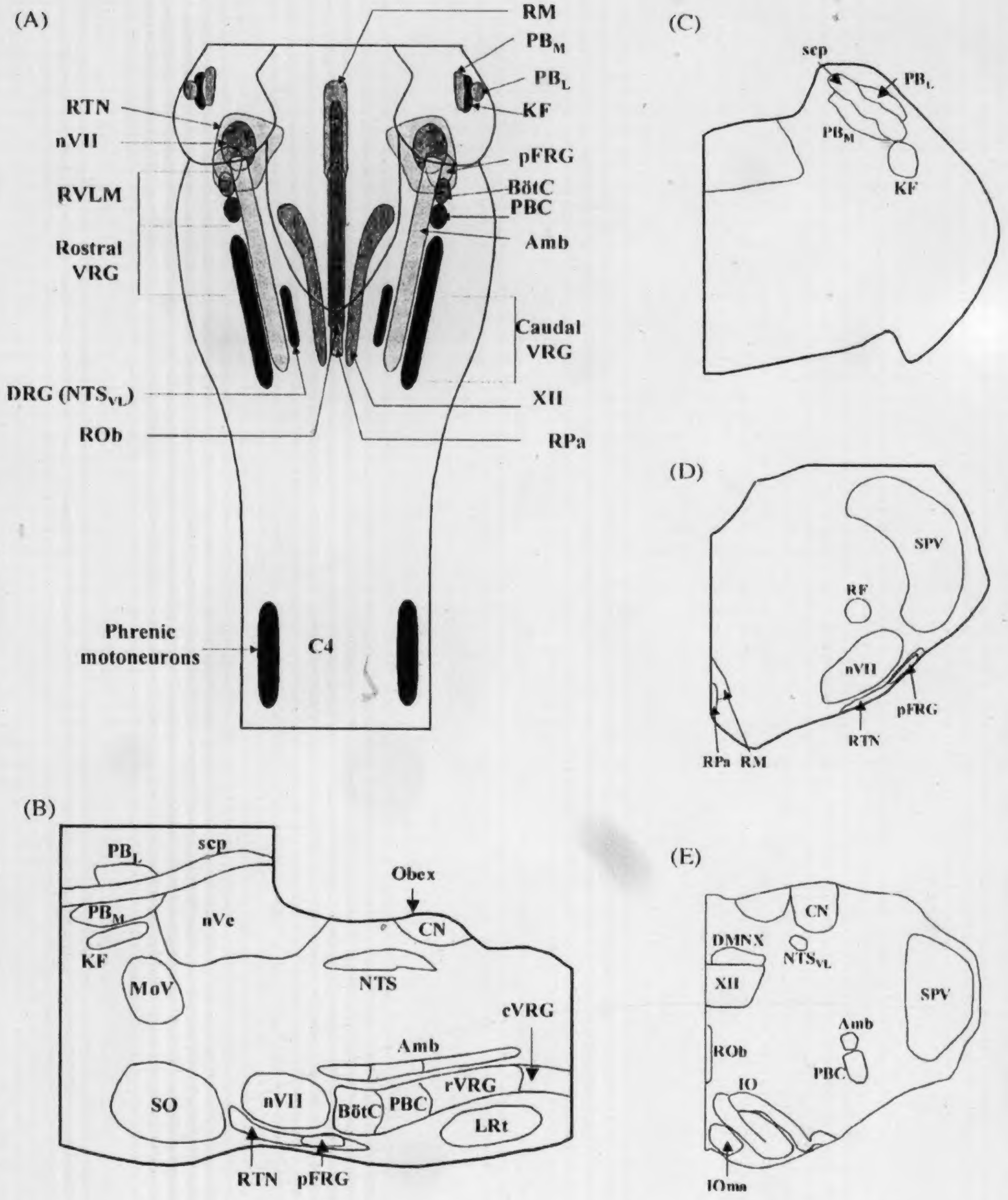
The respiratory stimulant acetazolamide, a carbonic anhydrase inhibitor prescribed to patients with pulmonary disease and to prevent acute mountain sickness (altitude sickness) has been used to alleviate CSA in patients with heart failure (Javaheri, 2006). Carbonic anhydrase is particularly abundant in tissues involved in breathing such as peripheral chemoreceptors, muscle, lung, red cells, glial cells and neurons (Teppema & Daham, 1999). Acetazolamide leads to metabolic acidosis that likely shifts the hypercapnic ventilatory response and lowers the $PaCO_2$ apnea threshold (White et al., 1982), via an increase in both central and peripheral chemosensitivity (Tojima et al., 1988; Coates et al., 1991). The effect of acetazolamide on the respiratory rhythm generator has not been determined. Long-term trials exploring the efficacy and safety of acetazolamide are not yet available.

Progesterone is a steroid hormone involved in the female menstrual cycle, pregnancy and embryogenesis of humans. Progesterone and other steroid hormones can act as chemical messengers in a wide range of species and target tissues including the brain, to produce a slow genomic response through intracellular nuclear receptor activation, and a rapid non-genomic response mediated by receptors associated with the cell membrane. Modulation of respiration by progesterone and other steroids has been widely investigated. *In vivo* and *in vitro* administration of progesterone stimulates ventilatory responses to hypoxia, primarily via the carotid body afferent signals to NTS or a direct effect on the central nervous system (Bayliss et al., 1987, 1990). Progesterone is an effective respiratory stimulant in a variety of physiological states including exercise and sleep (Skatrud et al., 1978). Clinical studies suggested that progestogens stimulates breathing in healthy male subjects and successfully used in patients with breathing disorders including OSA (Kimura et al., 1989). Medroxyprogesterone increases hypercapnic chemosensitivity and improves ventilation in patients with obesity hypoventilation syndrome and CSA (Block et al., 1981). However, most patients develop hypercapnia after progesterone therapy, suggesting that the efficacy of progesterone therapy is controversial (Kimura et al., 1989).

The methylxanthine derivates theophylline, aminophylline and caffeine derived from components of tea and coffee have multiple physiological and pharmacological mechanisms of action on stimulating respiration. Methylxanthines have been the mainstays of pharmacological treatment of apnea of prematurity since the 1970's and they are effective for the treatment of infants with recurrent apnea (Henderson-Smart, 2005). Methylxanthine therapy increases minute ventilation, improves CO_2 sensitivity, decreases hypoxic depression of breathing, enhances diaphragmatic activity, and decreases periodic breathing. Methylxanthines bind to specific adenosine recognition sites and block the actions of adenosine. Methylxanthines also inhibit cyclic nucleotide phosphodiesterases, the enzymes responsible for the hydrolytic inactivation of cyclic AMP and cyclic GMP (Howell et al., 1997). Methylxanthine therapy is employed to facilitate extubation of very low birth weight infants. However, research into the actions of adenosine and its receptors raises concerns about the safety of methylxanthine therapy in very preterm infants. Possible adverse effects include impaired growth, lack of neuroprotection during acute hypoxic-ischemic episodes, decreased cerebral blood flow, altered adenosine receptor-regulated behaviours and the responsiveness to adenosine agonists and abnormal behaviour (Hoecker et al., 2002; Millar & Schmidt, 2004; Pan & Chen, 2007). Thus, there is a call for the development of alternative therapeutic strategies.

1.4.3 OTHER APPROACHES

Other therapies, such as oral appliances, sleep positioning and palate surgery, should be considered for patients with OSA when CPAP is unsuccessful. Site-specific surgery, including maxillomandibular advancement, has been shown to be effective in selected patients with certain anatomical abnormalities (Prinsell, 1999). Tracheostomy is reserved for patients with severe OSA and cardiorespiratory compromise in cases where CPAP is neither tolerated nor effective. However, long-term tolerance of the oral appliances is generally not greater than the tolerance for CPAP. No studies have directly compared CPAP with palate surgery (e.g., uvulopalatopharyngoplasty). Although oxygen therapy (hyperoxia) was routinely used in varied clinical indications of central respiratory depression, no large-scale, long-term trials have been performed to determine which patients will likely benefit from O_2 therapy and its long-term efficacy. There are some concerns that O_2 therapy may have cardiodepressant effects mediated via O_2 radicals (Mak et al., 2001). A novel approach is supplementation of inspired air with a very low concentration of supplemental CO_2 to increase respiratory drive. While likely to be successful in decreasing apnea, it is doubtful that this would gain widespread acceptance (Martin & Abu-Shaweesh, 2005). Breathing techniques such as those used in Yoga encourage and strengthen nasal breathing, which allows easier, deeper and more relaxed inhalation. These breathing exercises may only be of some use in treating mild apnea and likely not useful for severe apneas.


(A)
RM
PB$_M$
PB$_L$
KF
RTN
nVII
pFRG
BötC
PBC
Amb
RVLM
Rostral VRG
Caudal VRG
DRG (NTS$_{VL}$)
XII
ROb
RPa
Phrenic motoneurons
C4
(B)
PB$_L$
scp
PB$_M$
KF
nVe
Obex
CN
NTS
MoV
cVRG
Amb
SO
nVII
BötC
PBC
rVRG
LRt
RTN
pFRG
(C)
scp
PB$_L$
PB$_M$
KF
(D)
SPV
RF
nVII
pFRG
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NTS$_{VL}$
XII
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Figure 1.1 Brainstem nuclei involved in the neural control of breathing. (A) Schematic longitudinal representation of brain stem nuclei those are associated with respiratory control. Amb, nucleus ambiguous; BötC, Bötzinger complex; DRG, dorsal respiratory group; KF, Kölliker-Fuse nucleus; NTS, nucleus tractus solitarius; RM, raphe magnus; PBC, pre-Bötzinger complex; PBL, lateral parabrachial nucleus; PBM, medial parabrachial nucleus; pFRG, parafacial respiratory group; RM, raphe magnus; ROb, raphe obscurus; RPa, raphe pallidus; RVLM, rostroventrolateral medulla; VRG, ventral respiratory group; XII, hypoglossal nucleus. For landmark reference: C4, cervical 4 level; nVII, facial nucleus. B. Schematic parasagittal representation of brain stem respiratory nuclei. LRt, lateral reticular nucleus; RTN, retrotrapezoid nucleus. For landmark reference: CN, cuneate nucleus; MoV, motor nucleus of trigeminal nerve; nVe, vestibular nuclei; SO, superior olivary nucleus. (C)–(E) Schematic representation of coronal sections of the rat brain stem at pontine (C), rostral medullary (D), and caudal medullary (E) levels. DMNX, dorsal motor nucleus of the vagus nerve; IO, inferior olivary nucleus; IOma, medial accessory olivary nucleus. For landmark reference: RF, retrofacial nucleus; scp, superior cerebellar peduncle; SPV, spinal nucleus of trigeminal nerve (from Wong-Riley & Liu, 2005).

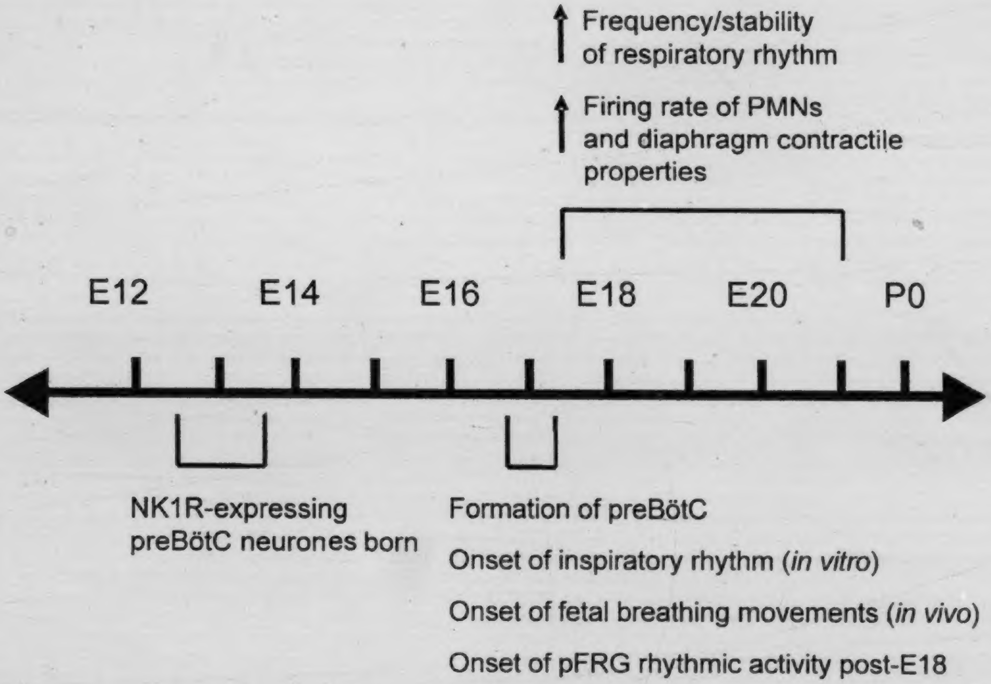
↑ Frequency/stability of respiratory rhythm
↑ Firing rate of PMNs and diaphragm contractile properties
E12
E14
E16
E18
E20
P0
NK1R-expressing preBötC neurones born
Formation of preBötC
Onset of inspiratory rhythm (*in vitro*)
Onset of fetal breathing movements (*in vivo*)
Onset of pFRG rhythmic activity post-E18

Figure 1.2 Time line illustrating key events in the development of respiratory neuronal activity in fetal rats (from Greer et al., 2006).

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*CHAPTER II

MODULATION OF RESPIRATORY RHYTHMOGENESIS BY CHLORIDE-MEDIATED CONDUCTANCES DURING THE PERINATAL PERIOD

*Previously published paper:

Ren J, Greer JJ (2006) Modulation of respiratory rhythmogenesis by chloride-mediated conductances during the perinatal period. J Neurosci 26(14):3721-30.

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2.1 INTRODUCTION

A primary goal of perinatal respiratory research is to identify the neurotransmitter systems responsible for modulating respiratory rhythmogenesis and motoneuron drive. Prenatally, an understanding of the neurochemical control of breathing *in utero* should provide insights into the mechanisms underlying episodic fetal breathing movements (FBMs) that are important for the maturation of lungs and respiratory neuromuscular systems (Kitterman, 1988; Harding & Hooper, 1996; Greer et al., 1999). Postnatally, the occurrence of central, obstructive and hypoxia-induced apnea in newborns is related to altered levels of neurochemical drive within medullary respiratory nuclei (Jansen & Chernick, 1991; Bonham, 1995; Ballanyi, 2004). The neurotransmitters, γ-aminobutyric acid (GABA) and glycine are the principal mediators of fast inhibitory transmission in the mammalian central nervous system. Synaptic inhibition mediated by GABA and glycine, while not essential for rhythm generation in the neonatal rodent (reviewed in Rekling & Feldman, 1998), strongly modulates mammalian respiratory rhythmogenesis and the patterning of motor output (Johnson et al., 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998; Ritter & Zhang, 2000). However, studies examining the function of GABA and glycine in respiratory control during the neonatal period have yielded equivocal and often contradictory results. Specifically, whether GABA_A and glycine receptor-mediated actions are depolarizing/hyperpolarizing resulting in stimulation/depression of respiratory frequency in neonates is unclear. In this study, we systematically investigated the effects of chloride-mediated conductances via GABA_A and glycine receptors on the generation of respiratory rhythm in newborn rats and in the fetus from the time of inception of fetal breathing movements. The potential role of taurine, an amino acid that acts via glycine receptors, was also examined. Taurine is in high concentrations within the medulla during the perinatal period (Sturman, 1993) and it has been associated with respiratory depression. Specifically, it reduces respiratory frequency when administered within the cerebral ventricles (Holtman et al., 1983) and taurine levels increase within the ventrolateral medulla during hypoxia-induced depression of newborn breathing (Hoop et al., 1999).

In vitro medullary slice and brainstem-spinal cord preparations isolated from perinatal rats were used to examine the effects of chloride-mediated conductances at the system (respiratory rhythm recorded from motor axons) and cellular (gramicidin perforated-patch recordings of respiratory neurons) levels. Complimentary data were obtained using plethysmographic recordings of unanesthetized rat pups.

2.2 MATERIAL AND METHODS

2.2.1 BRAINSTEM-SPINAL CORD & MEDULLARY SLICE PREPARATIONS

Fetal Sprague-Dawley (SD) rats (E17-21) were delivered from timed-pregnant dams anesthetized with halothane (2.5% delivered in 95% O_2 and 5% CO_2) and maintained at 37°C by radiant heat. The timing of pregnancies was determined from the appearance of sperm plugs in the breeding cages. The ages of fetuses were confirmed by comparison of their crown-rump length measurements with those published by Angulo Y González (1932). Newborn rats were anesthetized by inhalation of metofane (2-3%). Embryos and newborns were decerebrated, and the brainstem-spinal cord dissected following procedures similar to those established (Smith et al., 1990; Greer et al., 1992). The neuraxis was continuously perfused at 27 ± 1°C (perfusion rate of 5 ml/min; chamber volume of 1.5 ml) with modified Kreb's solution that contained the following (in mM): 128 NaCl, 3.0, 6.0 or 9.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 23.5 $NaHCO_3$, 0.5 NaH_2PO_4, and 30 D-glucose (equilibrated with 95%O_2-5%CO_2).

Details of the preparation have been described previously (Smith et al., 1991). Briefly, the brainstem-spinal cords isolated from perinatal rats as described above were pinned down, ventral surface upward, on a paraffin-coated block. The block was mounted in the vise of a vibratome bath (VT1000S; Leica, Nussloch, Germany). The brainstem was sectioned serially in the transverse plane starting from the rostral medulla to within approximately 150 μm of the rostral boundary of the preBötzinger complex (preBötC), as judged by the appearance of the inferior olive. A single transverse slice containing the preBötC and more caudal reticular formation regions was then cut (500-750 μm thick), transferred to a recording chamber, and pinned down onto a Sylgard elastomer. In some experiments, a N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) pH-buffered solution was used, where 24 mM HEPES replaced $NaHCO_3$ and NaH_2PO_4 (gassing with 100% O_2, pH adjusted to 7.3

with NaOH). Sodium was replaced with choline to examine the dependence of $[Cl^-]_i$ regulation on $[Na^+]_o$.

2.2.2 EXTRACELLULAR RECORDING AND ANALYSIS

Recordings of hypoglossal (XII) nerve roots and cervical (C4) ventral roots were made with suction electrodes. Suction electrodes were also placed into the XII nuclei and preBötC to record extracellular neuronal population discharge from medullary slice preparations. Signals were amplified, rectified, low-pass filtered, and recorded on a computer using an analog-to-digital converter (Digidata 1322A; Axon Inst., Foster City, CA) and data acquisition software (Clampfit, Axon). Mean values of respiratory frequency relative to control were calculated pre- and post-drug delivery. Values of EC_{50} and IC_{50} are defined as the concentration of drug necessary to produce 50% of the maximum mean measured response. Results were expressed as mean ± standard deviation (SD). Statistical significance was tested using paired/unpaired difference Student's *t* test; significance was accepted at *p* values <0.05.

2.2.3 INTRACELLULAR RECORDINGS FROM MEDULLARY SLICE PREPARATIONS

Patch electrodes were fabricated from thin-wall borosilicate glass (1.5 mm external and 1.12 mm internal diameter; A-M Systems, Everett, WA). The pipette resistances were between 3 and 5 MΩ. All whole-cell and perforated-patch recordings were obtained from the somata of small neurons within the preBötC, localized ventrolaterally to the pars compacta of the ambiguous nucleus. Neurons were approached under visual control using a microscope equipped with an infrared contrast enhancement system (Axioskop, Zeiss, Oberkochen, Germany). To establish whole-cell recording, additional suction was applied to rupture the underlying plasma membrane. Perforated-patch recordings were obtained using identical methods, except mechanical rupture of the plasma membrane was omitted. The progress of perforation was evaluated by monitoring the decrease in the membrane resistance. After the seal formation, series resistance decreased to 30-80 MΩ within 20-30 min. Gramicidin was dissolved in DMSO (1mg/100 μl, stock solution) and was freshly made every 2h. Stock solution (2-4 μl) was then added to 1 ml intracellular solution just before use. The pipette tips were pre-filled with 0.5 μl of gramicidin-free pipette solution to avoid contamination of tissue with gramicidin while searching for cells, and back filled with gramicidin-containing (20-40 μg/ml) pipette solution. The standard pipette solution contained the following (in mM): 140 K-gluconate, 4 NaCl, 1 $CaCl_2$, 10 EGTA or BAPTA, 10 HEPES, 5 MgATP, and 0.3 Na_3GTP, pH 7.3 with KOH.

Whole-cell recordings were initially established in modified Kreb's solution and performed with an NPI Electronics SEC05LX amplifier (NPI Electronics, Tamm, Germany). Neurons with resting membrane potentials (V_{rest}) more negative than -40 mV and overshooting action potentials were analyzed. Data were digitized with an analog-to-digital interface (Digidata 1322A; Axon Inst) and analyzed with the use of pClamp 9.2 (Axon).

2.2.4 PHARMACOLOGICAL AGENTS

All drugs were purchased from Sigma (St. Louis, MO) or RBI (Oakville, ON). Stock solutions of drugs were prepared as concentrates. All drugs used *in vitro* were dissolved in modified Kreb's solution and the pH adjusted to 7.4. Muscimol (soluble in physiological 0.9% NaCl saline; 0.5-1 $mgKg^{-1}$) and bicuculline (free base, soluble in DMSO, 0.6 $mgKg^{-1}$) were administered i.p. *in vivo*.

2.2.5 *IN VIVO* NEONATAL PLETHYSMOGRAPHIC MEASUREMENTS

Whole-body plethysmographic measurements of frequency and depth of breathing were made from P1 unanaesthetized rats of either sex. Pressure changes associated with perinatal rat breathing (produced by the warming and humidifying of inspired air and the subsequent cooling and condensation of expired air) were measured using a 27 ml whole-body plethysmograph chamber, a pressure transducer (model DP103, Validyne, Northridge, CA) and signal conditioner (CD-15). The plethysmograph was contained within an infant incubator (model C-86, Isolette, Warminster, PA) in order to maintain the ambient temperature at the approximate nest temperature of 32°C.

2.3 RESULTS

2.3.1 MODULATION OF RESPIRATORY FREQUENCY BY MUSCIMOL, GLYCINE AND TAURINE

Differential responses depending on type of *in vitro* preparation: Figs 2.1A-C show representative examples of the effects of muscimol ($GABA_A$ receptor agonist), glycine and taurine on the rhythmic respiratory discharge generated by brainstem-spinal cord (left column) and medullary slice (right column) preparations isolated from P1 rats. A very consistent pattern was observed. Activation of ligand-gated, chloride-mediated conductances resulted in a depression of respiratory frequency in brainstem-spinal cord preparations and an increase of frequency in medullary slice preparations. The population dose-response curves generated from 24 brainstem-spinal cord and 22 medullary slice preparations are presented in Figs 2.1D-F. The muscimol-induced modulation of respiratory frequency generated by brainstem-spinal cord (IC_{50} = 0.31 μM) and medullary slice (EC_{50} = 0.25 μM) preparations were antagonized by bicuculline (3 μM), but not by strychnine (1 μM). The glycine-induced inhibition of brainstem-spinal cord (IC_{50} = 36 μM) and increase of medullary slice (EC_{50} = 34 μM) respiratory frequencies were antagonized by strychnine (1 μM), but not by bicuculline (3 μM). The taurine-induced inhibition of brainstem-spinal cord (IC_{50} = 0.59 mM) and increase of medullary slice (EC_{50} = 0.46 mM) respiratory frequencies were antagonized by strychnine (1 μM), but not by bicuculline (3 μM). Respiratory frequency was not significantly altered from control values in either preparation by addition of low doses of strychnine or bicuculline on their own. These results demonstrate that the muscimol-induced responses were mediated through $GABA_A$ receptors and the taurine/glycine-induced responses were mediated through glycine receptors.

The next series of experiments examined the effects of increasing the endogenous levels of GABA on respiratory frequency in brainstem-spinal cord and medullary slice preparations. Fig 2.2A shows representative examples of the effects of bath application of the GABA-uptake inhibitor nipecotic acid (2 mM) to brainstem-spinal cord (left column) and medullary slice (right column) preparations from P1 rats. Population data generated from 5 brainstem-spinal cord and 4 medullary slice preparations are shown in Fig 2.2B. In the brainstem-spinal cord, there was a clear decrease in respiratory frequency after 20 minutes of nipecotic acid application. An antagonist to the GABA$_B$ receptor, saclofen (400 μM), was added to the bathing medium to discern the component of the respiratory rhythm suppression resulting from the actions via GABA$_B$ receptors. These data show that a portion of the respiratory suppression was due to GABA$_B$ receptor-mediated action. Subsequently, bicuculline (3 μM) was added to the bathing medium, demonstrating that a significant component of the respiratory frequency depression was due to actions via GABA$_A$ receptors. In medullary slice preparations, there was a slight, but statistically insignificant, increase in respiratory frequency after 20 minutes of nipecotic acid application. However, there was a significant increase in respiratory frequency after the administration of saclofen (400 μM). This indicates that activation of GABA$_A$ receptors on their own caused an increase in respiratory frequency in medullary slice preparations. This increase in frequency was antagonized by bicuculline.

$[K^+]_o$ dependency of responses to chloride-mediated conductances: As demonstrated above, activation of ligand-gated, chloride-mediated conductances consistently resulted in a decrease and increase of respiratory frequency in brainstem-spinal cord and medullary slice preparations, respectively. We investigated two possible mechanisms for this seemingly paradoxical finding. First, we tested the hypothesis that the differential responses were due to the indirect actions of other neuromodulators that were present to varying degrees in the two types of *in vitro* model. The preparation-dependent differential responses persisted in the presence of antagonists to μ-opioid (naloxone, 10 μM; n=5), NMDA (MK-801, 30 μM; n=4), α1 (prazosin, 10 μM; n=4), α2 (idazoxan, 10 μM; n=3), nicotinic (d-tubocurarine, 5 μM; n=3) or muscarinic (atropine, 1 μM; n=3) receptors, suggesting that indirect modulatory actions were not responsible. Second, we examined whether the markedly different $[K^+]_o$ in the solutions bathing the brainstem-spinal cord and medullary slice preparations could account for the differential responses. Brainstem-spinal cord preparations produce a robust respiratory rhythm when bathed in physiological levels of 3 mM $[K^+]_o$. In contrast, medullary slice preparations are typically bathed in 9 mM $[K^+]_o$ to promote the production of robust respiratory discharge for extended periods (i.e. hours). Thus, we performed experiments on both types of preparations isolated from P2 rats with varying $[K^+]_o$. It should be noted that spontaneous rhythmic activity persisted for up to 2 hours in some P2 medullary slice preparations cut to a thickness of 750 µm, which allowed for testing of bath application of muscimol. Figs 2.3A,B illustrate representative examples of the effects of muscimol on respiratory frequency in P2 brainstem-spinal cord and medullary slice preparations perfused with medium containing either 3, 6 or 9 mM $[K^+]_o$. Population data generated from brainstem-spinal cord and medullary slice preparations (n=5 each) are presented in Fig 2.3C. Muscimol caused a suppression, no effect, or an increase in respiratory frequency when brainstem-spinal cord or medullary slice preparations were perfused with 3, 6 or 9 mM $[K^+]_o$, respectively. These results demonstrate that muscimol-induced effects are dependent on the $[K^+]_o$ of the bathing media rather than the type of *in vitro* preparation *per se*.

***In vivo* plethysmoghraphic measurements from neonatal rats:** We complimented the *in vitro* experiments with whole-body plethysmographic measurements of frequency and depth of breathing of P1 rats (n = 5) before and after i.p. injection of $GABA_A$ receptor agonist muscimol (0.5-1 mg/kg). There was a marked suppression of respiratory frequency similar to that observed with *in vitro* preparations bathed in 3 mM $[K^+]_o$. The muscimol-induced suppression of respiratory frequency was antagonized by a dose of bicuculline (0.6 mg/kg) that had no significant effects on baseline breathing on its own.

2.3.2 DEVELOPMENTAL CHANGES IN THE ACTIONS OF CHLORIDE-MEDIATED CONDUCTANCES

We next examined the age-dependent changes of the actions of chloride-mediated conductances during the perinatal period. The inception of respiratory rhythm occurs at approximately E17 in rats (Greer et al., 1992; DiPasquale et al., 1996; Kobayashi et al., 2001; Pagliardini et al., 2005) and thus that was the earliest age examined. Fig 2.4A shows representative traces of respiratory neuronal discharge recorded from E17, E18 and E20 brainstem-spinal cord preparations in response to bath application of muscimol (0.3 μM). Respiratory frequency was increased at E17, not significantly changed at E18 and decreased at E20 by muscimol. Population data generated from 61 brainstem-spinal cord and 65 medullary slice preparations are presented in Fig 2.4B. In medullary slice preparations perfused with 9 mM $[K^+]_o$, muscimol increased respiratory frequency from E17-P5. The suppression of respiratory frequency by muscimol was particularly pronounced in E20 and E21 brainstem-spinal cord preparations.

2.3.3 $[K^+]_o$ AND AGE DEPENDENT EFFECTS ON THE CHLORIDE EQUILIBRIUM POTENTIAL (E_{Cl^-})

Perforated-patch recordings of respiratory neurons within the region of the preBötC under various $[K^+]_o$ conditions and at different developmental ages were performed. Note that $[Cl^-]_i$ is stable with gramicidin perforated recordings (Kyrozis and Reichling, 1995) as opposed to conventional whole-cell recording that leads to a very rapid dialysis of $[Cl^-]_i$ (Marty and Neher 1995).

Muscimol application: Fig 2.5A shows a perforated-patch recording from an inspiratory (I) neuron in a P2 medullary slice preparation bathed in 9 mM (left panel] or 3 mM (right panel) $[K^+]_o$. Muscimol (0.3 μM) evoked a depolarizing response from V_{rest} of -52 mV with 9 mM $[K^+]_o$ and an increase of respiratory frequency. After switching to 3 mM $[K^+]_o$, the membrane potential (V_m) hyperpolarized and stabilized at -61 mV within 5 minutes. Subsequent application of muscimol (0.3 μM) hyperpolarized V_m and decreased respiratory frequency. Fig 2.5B demonstrates the reversal potential at P1 for the $GABA_A$ receptor-activated membrane response (E_{GABA-A}) of an I neuron using perforated-patch recording in the presence of TTX (0.3 μM). In 9 mM $[K^+]_o$, (left panel), application of muscimol (3 μM, 30 s) evoked a hyperpolarizing response from a holding potential (V_h) of -36 mV, while it depolarized the neuron from V_h of -52 mV and -88 mV. V_{rest} of this neuron in 9 mM $[K^+]_o$ was -52 mV. In 3 mM $[K^+]_o$, the application of muscimol (3 μM) evoked a hyperpolarizing response from V_h of -36 mV and -52 mV, while it depolarized the neuron from a V_h of -88 mV. V_{rest} was -60 mV when superfused with 3 mM $[K^+]_o$. A linear regression line (Fig 2.5C) was calculated for the amplitudes of muscimol-evoked responses from the neuron shown in (B) to determine E_{GABA-A}. Fig 2.5D shows the population data for E_{GABA-A} and V_{rest} at 3 mM versus 9 mM $[K^+]_o$ at different stages of development for 49 I neurons. In summary, a shift in $[K^+]_o$ from 3 to 9 mM caused an 8mV depolarization of V_{rest}, a 10-20% increase in membrane input resistance at all ages from E17-P4, an 11 mV depolarization of E_{GABA-A} at E17-E18, and a 17 mV depolarization of E_{GABA-A} from E20-P4.

Fig 2.6A illustrates a gramicidin perforated-patch recording of an I neuron in a P1 medullary slice bathed in solution containing 9 mM $[K^+]_o$ and 0.3 μM TTX. There was a dose–dependent depolarization (from V_{rest} of -50 mV) and reduction in input resistance in response to muscimol application. The population dose-response data for 5 I neurons is shown in Fig 2.6B. Bicuculline (3 μM) antagonized the depolarizing actions of lower doses of muscimol. The effects of relatively high doses of muscimol (3 μM), used in past studies, on V_m of an I neuron and XII nerve root respiratory activity at P1 is shown in current- (Fig 2.6C) and voltage-clamp (Fig 2.6D) modes. Muscimol application caused a depolarization of V_m and an inward current. There was an initial increase in the frequency of inspiratory discharge followed by suppression of spiking in the neuron and the amplitude of the XII nerve recording. The combination of sodium channel inactivation and shunting of inward glutamatergic currents that occurs with higher concentrations of muscimol may abolish rhythmic respiratory discharge.

Bicarbonate efflux through $GABA_A$ receptor-coupled Cl^- channels can produce a considerable depolarizing shift of E_{GABA-A} (Kaila, 1994). To test whether membrane diffusion of bicarbonate substantially contributed to $GABA_A$ receptor-mediated responses, the muscimol effect was tested in CO_2/HCO_3 free bath solution (HEPES-buffered solution; 9 mM $[K^+]_o$) in P2 rats. Perfusion of CO_2/HCO_3-free

HEPES-buffered saline did not affect V_{rest} (-51 ± 1.8 mV, n=4). Subsequent application of muscimol evoked a depolarization and E_{GABA-A} was -46.2 ± 2.0 mV (n=4). Thus, as reported previously (Ritter and Zhang, 2000), E_{GABA-A} observed using *in vitro* medullary slice preparations are largely independent of bicarbonate conductances.

Taurine and glycine application: Fig 2.7 illustrates the influence of $[K^+]_o$ on taurine- and glycine-induced responses. The response to application of taurine (5 mM) of an I neuron in a P1 medullary slice bathed in 3 mM or 9 mM $[K^+]_o$ and 0.3 µM TTX is shown in Fig 2.7A. V_{rest} was -59 mV and -51 mV in 3 mM and 9 mM $[K^+]_o$, respectively. In 3 mM $[K^+]_o$, taurine caused an outward current from V_h of -25 mV and -55 mV and an inward current from a V_h of -90 mV. In 9 mM $[K^+]_o$, taurine caused an outward current from a V_h of -25 mV and inward currents from V_h of – 55 mV and -90 mV. Current-voltage relationship of $I_{taurine}$ was fitted by a linear regression line (Fig 2.7B). The population data for the taurine-evoked responses in 5 I neurons are shown in Fig 2.7C. In 3 mM $[K^+]_o$, V_{rest} and $E_{taurine}$ were -58.8 ± 1.6 mV and -63.5 ± 2.3 mV, respectively. In 9 mM $[K^+]_o$, V_{rest} and $E_{taurine}$ were -50.1 ± 1.4 mV and -46.2 ± 1.7 mV, respectively. Similarly, $E_{glycine}$ was -46.5 ± 1.5 mV, and -64.2 ± 2.2 mV when preparations were perfused with 9 mM and 3 mM $[K^+]_o$, respectively (n=4; Fig 2.7D). There were no significant differences in the reversal potential among taurine-, glycine- or muscimol-induced responses.

2.3.4 INFLUENCE OF $[K^+]_o$ ON THE REVERSAL POTENTIAL OF IPSPs (E_{IPSP})

Inhibitory post-synaptic potentials (IPSPs) were studied from recordings of expiratory (E) neurons in neonatal medullary slice preparations. Fig 2.8A shows recordings from an E cell in a P1 rat preparation using both gramicidin perforated-patch and conventional whole-cell recordings. V_{rest} was -49 mV with both recording techniques. The IPSPs during the inspiratory phase are accentuated during whole-cell recordings when $[Cl^-]_i$ is decreased by dialysis of the pipette solution. Fig 2.8B shows that strychnine (1 μM), but not bicuculline (30 μM), blocked inspiratory-related IPSPs of an E neuron recorded from a P1 rat with the conventional whole-cell recording. This observation is consistent with the report by Shao and Feldman (1997) showing glycinergic-mediated IPSPs in the preBötC. E_{IPSP} from a tonic E-cell with perforated-patch recordings was measured in 9 mM (Fig 2.8C) and 3 mM (Fig 2.8D) $[K^+]_o$. V_{rest} and E_{IPSP} in 9 mM $[K^+]_o$ was -51 mV and between -42 and -51 mV, respectively. V_{rest} and E_{IPSP} in 3 mM $[K^+]_o$ was -61 mV and between -61 and -70 mV, respectively. Fig 2.8E shows the linear regression line calculated for the amplitudes of IPSPs from Figs 2.8C and D. Population data for 5 E neurons is presented in Fig. 8F. Similar to that observed from I cells, there was an overall shift in E_{IPSP} of approximately 18 mV in the hyperpolarizing direction when the bathing solution was switched from 9 to 3 mM $[K^+]_o$.

2.3.5 PERTURBATIONS OF CHLORIDE TRANSPORTER FUNCTION

Neuronal $[Cl^-]_i$ is regulated by the action of two principal cation-chloride co-transporters. Typically, the Na-K-2Cl co-transporter (NKCC1) raises $[Cl^-]_i$ and the K-Cl co-transporter (KCC2) lowers $[Cl^-]_i$ (Kaila, 1994). We examined how perturbation of each of these chloride co-transporters affected muscimol-induced responses of respiratory neurons in medullary slice preparations of varying perinatal age and $[K^+]_o$.

Effects of removing extracellular sodium ions: The NKCC1 transporter is expressed early in neuronal development and plays a role in increasing $[Cl^-]_i$ (Payne et al., 2003). We impaired the function of the NKCC1 transporter by replacing $[Na^+]_o$ with choline. All recordings were performed in 3 mM $[K^+]_o$ Fig 2.9 A shows that application of muscimol (3 μM for 30seconds) induces a depolarization from V_h of -50 mV and -65 mV in a perforated-patch recording of an I neuron at E17 in control conditions (left panels). $E_{GABA\text{-}A}$ was shifted close to V_{rest} after removal of extracellular Na^+ (middle panels). The effect was reversible after return to control solution (right panels). Population data from 4 I neurons are shown in Fig 2.9C. In contrast, we did not observe a significant change in $E_{\text{GABA-A}}$ from ages P1-P3 after replacing $[\text{Na}^+]_o$ with choline (data not shown).

Effects of bumetanide application: Administration of muscimol (0.3 μM) caused an increase in the respiratory frequency generated by E17 brainstem-spinal cord and medullary slice preparations (Fig 2.10A, top traces). The muscimol-induced excitatory effects were blocked by 20-40 minute bath application of bumetanide (10 μM), a specific inhibitor of the NKCC1 co-transporter (Fig 2.10A, bottom traces). At P2, the respiratory frequency generated by brainstem spinal cord and medullary slice preparations was decreased and increased by application of muscimol (0.3 μM), respectively (Fig 2.10B, top traces). Neither of the muscimol-induced changes was modified by bumetanide (10 μM; Fig 2.10B, bottom traces). Population data for the actions of muscimol and muscimol plus bumetanide for both types of E17 *in vitro* preparations (n = 5) are presented in Fig 2.10C. Perforated-patch recordings were made from 6 I neurons in E17 medullary slice preparations bathed in 3 mM $[\text{K}^+]_o$ in the absence and presence of bumetanide (10 μM). After exposure to bumetanide, $E_{\text{GABA-A}}$ shifted from -42.3 ± 2.5 mV to 51.8 ± 3.7 mV without a significant change in V_{rest} (Fig 2.10D).

Effects of furosemide application: Application of furosemide was used to block the KCC2 co-transporter in brainstem-spinal cord and medullary slice preparations at P1-P2. The muscimol-induced suppression of respiratory frequency generated in the brainstem-spinal cord was blocked after 25 minute exposure to furosemide (1 mM; Fig 2.11A). The muscimol-induced depolarization of an I neuron and increase in respiratory frequency in a medullary slice preparation bathed in 9 mM $[\text{K}^+]_o$ was attenuated by furosemide (1 mM; Fig 2.11B). The population data for the effects of muscimol and muscimol plus furosemide on respiratory frequency generated by neonatal *in vitro* preparations are shown in Fig 2.11C. The population data showing the effects of furosemide on 6 I neurons in presented in Fig 2.11D. Furosemide did not significantly affect V_{rest} of neurons bathed in either 3 mM or 9 mM $[\text{K}^+]_o$. However, furosemide significantly shifted $E_{\text{GABA-A}}$ from -46.2 ± 2.0 mV to

-49.5 ± 2.3 mV and -64.1 ± 1.5 mV to -59.6 ± 2.7 in 9 mM and 3 mM $[K^+]_o$, respectively. The muscimol (0.3 μM) -induced increase of respiratory frequency at E17 persisted in the presence of furosemide (1 mM, data not shown). It should be noted that furosemide also affects NKCC1 co-transporter function (Gillen et al., 1996; Payne et al., 2003). However, as discussed above, a perturbation of the NKCC1 transporter with removal of $[Na^+]_o$ or bumetanide did not affect muscimol-induced responses during the postnatal period and thus the data can be explained by a specific interference with KCC2 function.

2.4 DISCUSSION

These data demonstrate the age-dependent changes in the effects of chloride-mediated conductances on respiratory frequency from the time of inception of fetal inspiratory drive through to the newborn period. The transition from an excitatory to inhibitory effect on respiratory neurons and rhythmogenesis occurred at approximately E19. GABA, glycine and taurine all suppressed respiratory frequency by birth. The actions of chloride-mediated conductances on respiratory activity are profoundly affected by $[K^+]_o$ which explains some of the discrepancies from past studies using different *in vitro* models.

2.4.1 DEPENDENCE OF CHLORIDE-MEDIATED CONDUCTANCES ON $[K^+]_o$

Our initial observation that muscimol, glycine and taurine caused contrasting changes in respiratory frequency between neonatal brainstem-spinal cord and medullary slice preparations was unexpected. Subsequent experimentation demonstrated that the apparent discrepancy could be accounted for by different levels of $[K^+]_o$ in the media used to bathe the two types of *in vitro* preparations. Specifically, $[K^+]_o$ influenced the function of co-transporters that determine transmembrane Cl^- gradients (discussed below). Perforated patch recordings from both I and E neurons demonstrated that increasing $[K^+]_o$ from 3 to 9 mM results in an approximate 8 mV depolarization of V_{rest} and a 15-20 mV depolarization (E20-P4) of the reversal potential for chloride-mediated conductances. Thus, elevating the $[K^+]_o$ to 9 mM in either type of postnatal *in vitro* preparation caused an efflux rather than an influx of Cl^- seen with 3 mM $[K^+]_o$ in response to $GABA_A$ or glycine receptor agonists. These data can explain the inconsistencies from past studies examining the actions of chloride-mediated conductances on respiratory rhythm in neonatal rodent *in vitro* preparations. Brockhaus and Ballanyi (1998) reported predominantly hyperpolarizing actions of $GABA_A$ and glycine receptor-mediated conductances in newborn brainstem-spinal cord preparations (bathed in 3 mM $[K^+]_o$). Further, Fregosi et al.

(2004) reported a depression of respiratory frequency generated by brainstem-spinal cord preparations in response to $GABA_A$ receptor activation. In contrast, Ritter and Zhang (2000) reported predominantly depolarizing actions in medullary slice preparations derived from newborn mice (bathed in 9 mM $[K^+]_o$). We propose that differences in results and thus interpretation of the actions of chloride-mediated conductance were due in part to altered $[K^+]_o$ conditions.

2.4.2 DEPENDENCE OF CHLORIDE-MEDIATED CONDUCTANCES ON PERINATAL STAGE OF DEVELOPMENT

Respiratory rhythmogenesis within the preBötC commences on E17 in the rat (Pagliardini et al., 2003). Prior to E17, there is a robust endogenous embryonic rhythmic activity present throughout much of the developing neuraxis, including the ventrolateral medulla (Greer et al., 1992; Ren and Greer, 2003; Ren et al., 2006). GABA and glycine act as excitatory neurotransmitters promoting the emergence of embryonic rhythms (Ren and Greer, 2003). Data from this study demonstrates that agonists to $GABA_A$ and glycine receptors also lead to a depolarization of I neurons and an increase in respiratory frequency at the inception of fetal respiratory drive. The switch from excitatory to inhibitory actions of chloride-mediated conductances is at approximately E19. By E20-21, activation of $GABA_A$ or glycine receptors (via glycine or taurine) results in a hyperpolarization of respiratory neurons and depression of respiratory frequency *in vitro* under typical physiological levels of $[K^+]_o$. The *in vivo* data demonstrating a suppression of respiratory rhythm after i.p. administration of muscimol to rat pups are consistent with those data.

Functionally, the depolarization of respiratory neurons at early stages of development will remove NMDA voltage-dependent Mg^{2+} block and thus enhance glutamate-mediated depolarization and rise of $[Ca^{2+}]_i$ (Rohrbough and Spitzer, 1996; Ziskind-Conhaim, 1998). In turn, elevated $[Ca^{2+}]_i$ regulates neurite outgrowth, gene expression, transmitter release and local receptor protein aggregation (Ben Ari et al., 1997; Yuste and Katz, 1991). Collectively, these factors could facilitate the marked phenotypic changes of electrophysiological and firing properties of VRG neurons that occur between E17-E19 (DiPasquale et al., 1996). Later in gestation and in the newborn period, elevated levels of GABA, glycine or taurine (e.g. in response to hypoxia) within the preBötC will result in a suppression of respiratory frequency.

2.4.3 DEPENDENCE OF CHLORIDE-MEDIATED CONDUCTANCES ON CHLORIDE CO-TRANSPORTER FUNCTION

Early in fetal development, the NKCC1 is expressed at relatively high levels and thus there is an elevated intracellular [Cl^-] relative to mature neurons (Kaila, 1994). The increased expression of the KCC2 co-transporter with perinatal age leads to the extrusion of Cl^- from the cytoplasm and thus establishment of an E_{Cl^-} that is hyperpolarized from Vrest (Rivera et al., 1999). Perturbation of NKCC1 function with $[Na^+]_o$-free solution and bumetanide shifted E_{Cl^-} to hyperpolarized values in pre-E18 in vitro preparations. The shift in E_{Cl^-} was sufficient to reverse the normal muscimol-induced increase in respiratory frequency. Perturbations of KCC2 function with furosemide had no significant effect pre-E18. Those data are consistent with the dominance of NKCC1 function that results in E_{Cl^-} at values less negative than Vrest. In contrast, perturbation of KCC2 function with furosemide in neonatal preparations resulted in the block of muscimol-induced decrease in respiratory frequency in the brainstem-spinal cord preparations and hyperpolarization of E_{Cl^-} in the medullary slice perfused with 3mM $[K^+]_o$ solution. Block of NKCC1 function had no significant effect. Thus the function of KCC2 extrusion of Cl^- dominated control of E_{Cl^-} postnatally. Further, perturbation of KCC2 function with furosemide in neonatal preparations resulted in the block of muscimol-induced increase in respiratory frequency and depolarization of E_{Cl^-} in the medullary slice perfused with 9 mM $[K^+]_o$ solution. These data indicate that changes in KCC2 function in elevated $[K^+]_o$ were responsible for a significant component of the differential responses to chloride-mediated conductances observed in bathing mediums with different $[K^+]_o$. As previously demonstrated in neocortical pyramidal neurons, there is an > 15 mV depolarization of the E_{Cl^-} with a change from 3.5 to 10 mM extracellular $[K^+]$

(DeFazio et al., 2000). These results confirmed the observation that KCC2 could extrude or accumulate Cl^- depending on $[K^+]_o$ (Payne 1997).

2.4.4 SUMMARY

Respiratory neurons in the ventrolateral medulla are depolarized by chloride-mediated conductances prior to, and at the time of inception, of respiratory rhythmogenesis in the fetal rat. By E19, chloride-mediated conductances induce a hyperpolarization of respiratory membrane potential and suppression of respiratory frequency. The action of chloride-mediated conductances is determined by the ontogenesis of chloride co-transporters. The function of chloride co-transporters is strongly modulated by $[K^+]_o$ and this must be considered when evaluating responses observed using *in vitro* perinatal preparations.

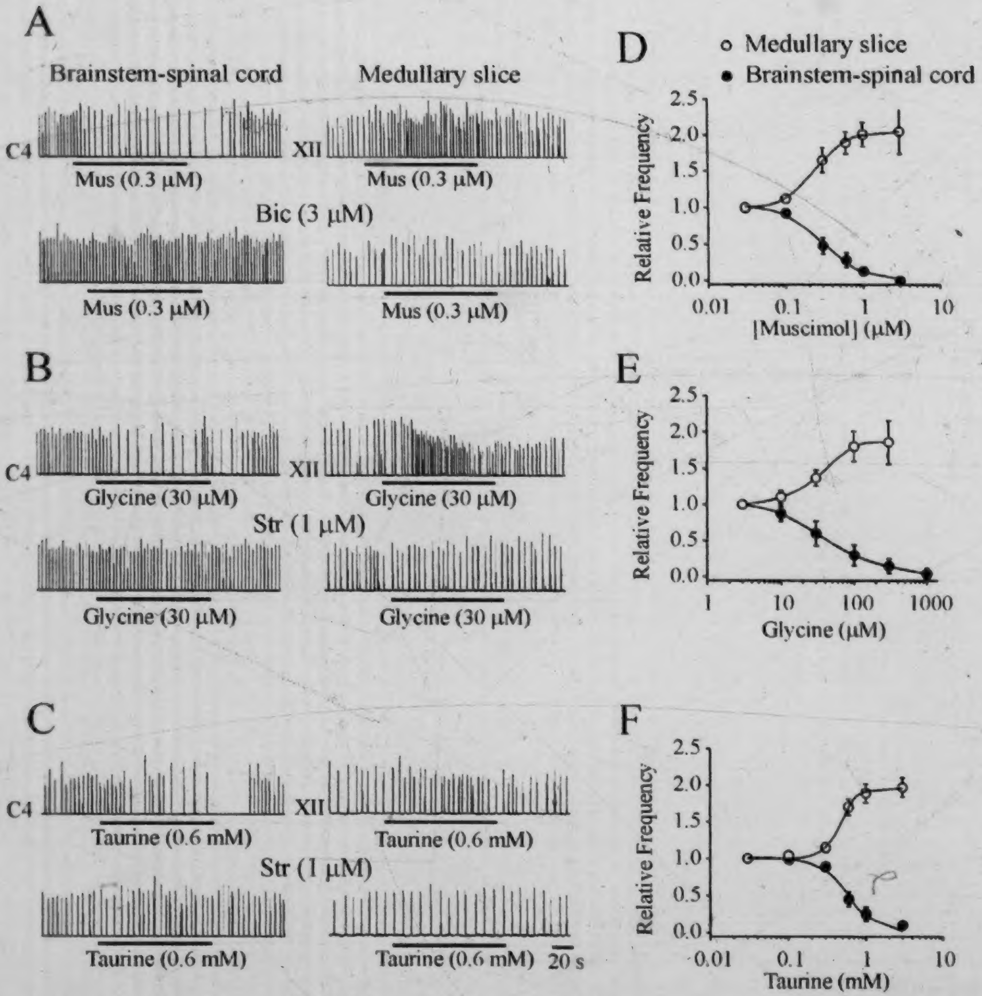


Figure 2.1 Effects of chloride-mediated conductances on respiratory rhythm generated by brainstem-spinal cord and medullary slice preparations isolated from postnatal (P)1 rats. Rectified and integrated suction electrode recordings of C4 ventral roots (brainstem-spinal cord) and XII nerve roots (medullary slice) in response to bath application of the receptor agonists (A) muscimol (Mus), (B) glycine and (C) taurine (applied during the time indicated by solid line). The actions of muscimol were antagonized by bicuculline (Bic). The actions of glycine and taurine were antagonized by strychnine (Str). D-F) Population dose-response data for each of the agonists added to the media bathing both types of *in vitro* preparations (n=5-6). Brainstem spinal cord and medullary slice preparations were bathed in 3 mM and 9 mM $[K^+]_o$, respectively.

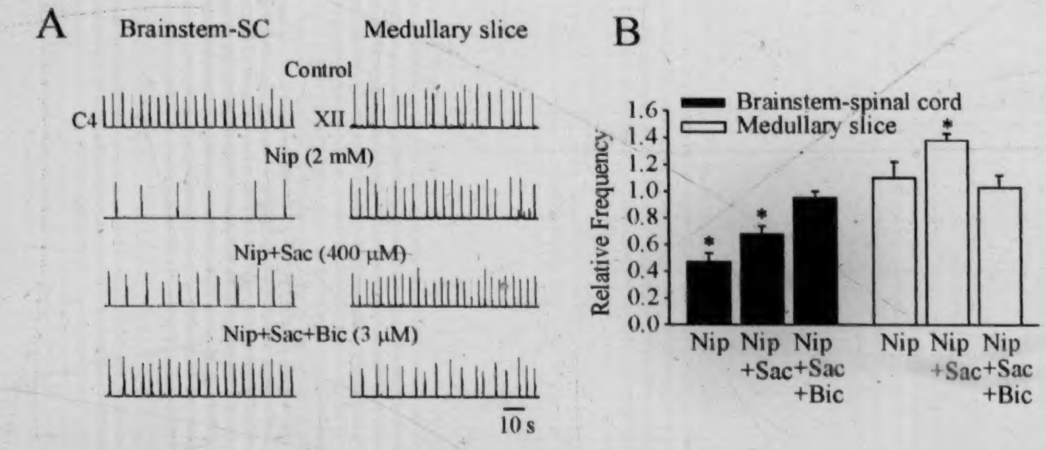


Figure 2.2 Effects of increasing the levels of endogenously released GABA in P1 brainstem-spinal cord (SC) and medullary slice preparations by bath application of the uptake inhibitor nipecotic acid (Nip). A) Representative recordings from brainstem-spinal cord (left column) and medullary slice (right column) preparations. Nipecotic acid caused a clear decrease in respiratory frequency in the brainstem-spinal cord preparation with 20 minutes of application. A part of the inhibition was removed in the presence of the GABA$_B$ receptor antagonist saclofen (Sac). The remainder of the inhibition was removed by the addition of the GABA$_A$ receptor antagonist bicuculline (Bic). In medullary slice preparations, there was no significant change in respiratory frequency in response to application of nipecotic acid. However, there was a significant increase in respiratory frequency by the subsequent application of saclofen. The increase in respiratory frequency was blocked by bicuculline, demonstrating that the major component of the excitatory action was via GABA$_A$ receptors. (B) Population data for from bath application of drugs in brainstem-spinal cord and medullary slice preparations. * indicates significant difference relative to control (*p* values <0.05). Brainstem spinal cord and medullary slice preparations were bathed in 3 mM and 9 mM [K$^+$]$_o$, respectively.

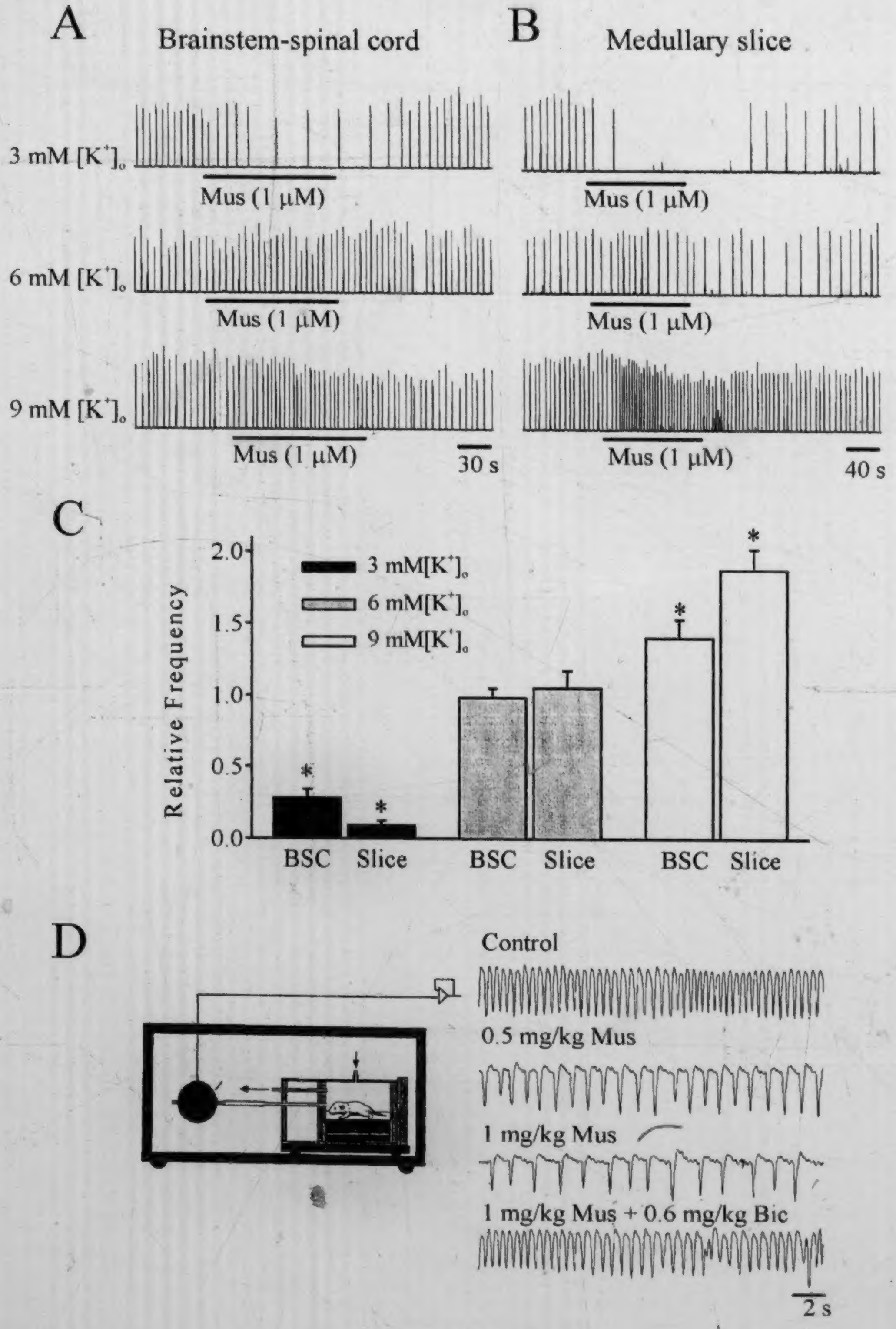

A
Brainstem-spinal cord
B
Medullary slice
3 mM [K⁺]ₒ
Mus (1 μM)
Mus (1 μM)
6 mM [K⁺]ₒ
Mus (1 μM)
Mus (1 μM)
9 mM [K⁺]ₒ
Mus (1 μM)
Mus (1 μM)
30 s
40 s
C
3 mM[K⁺]ₒ
6 mM[K⁺]ₒ
9 mM[K⁺]ₒ
Relative Frequency
2.0
1.5
1.0
0.5
0.0
*
*
*
*
BSC
Slice
BSC
Slice
BSC
Slice
D
Control
0.5 mg/kg Mus
1 mg/kg Mus
1 mg/kg Mus + 0.6 mg/kg Bic
2 s

Figure 2.3 $[K^+]_o$ dependency of responses to chloride-mediated conductances. The effects on respiratory frequency of muscimol in P2 (A) brainstem-spinal cord and (B) medullary slice preparations bathed in 3, 6 of 9 mM $[K^+]_o$. Muscimol (1 μM) decreased respiratory frequency when the brainstem-spinal cord or medullary slice preparations were superfused with 3 mM $[K^+]_o$ bathing solution. On the contrary, muscimol (1 μM) increased respiratory frequency when the same preparation was superfused with 9 mM $[K^+]_o$ bathing solution. Muscimol (1 μM) had no significant effect on respiratory frequency when the same preparation was superfused with 6 mM $[K^+]_o$. C) Population data for responses to muscimol of brainstem-spinal cord (BSC) and medullary slice (slice) preparations bathed in media with different $[K^+]_o$. D) The effects of i.p. administration of two doses of muscimol to an unanesthetized P2 rat pup. Respiratory frequency was measured using a whole-body plethysmograph contained within an infant incubator for temperature control. The muscimol-induced suppression of respiratory frequency was antagonized by bicuculline. * indicates significant difference relative to control (p values <0.05).

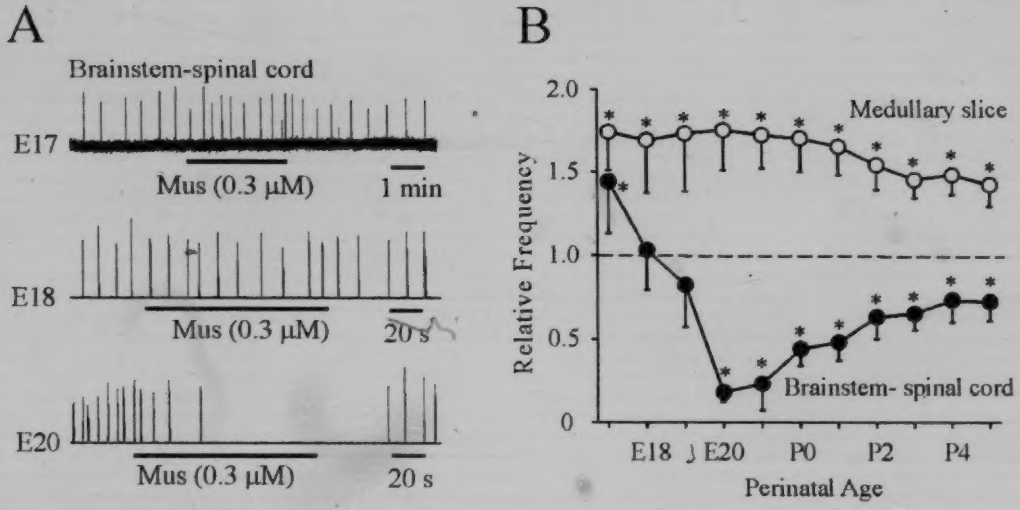


Figure 2.4 Age-dependent changes in the effects of chloride-mediated conductances. A) Rectified and integrated suction electrode recordings of XII nerve roots of brainstem-spinal cord preparations bathed in 3 mM $[K^+]_o$ during the perinatal period. Muscimol caused an increase, no significant change and decrease of respiratory frequency at ages E17, E18 and E20, respectively. B) Population data for changes in respiratory frequency relative to control of medullary slice and brainstem spinal cord preparations in response to bath application of muscimol. The transition from an excitatory to inhibitory action in brainstem-spinal cord preparations occurred at approximately E19. Respiratory frequency increased in medullary slice preparations bathed in 9 mM $[K^+]_o$ at all ages. * indicates significant difference relative to control (p values <0.05).

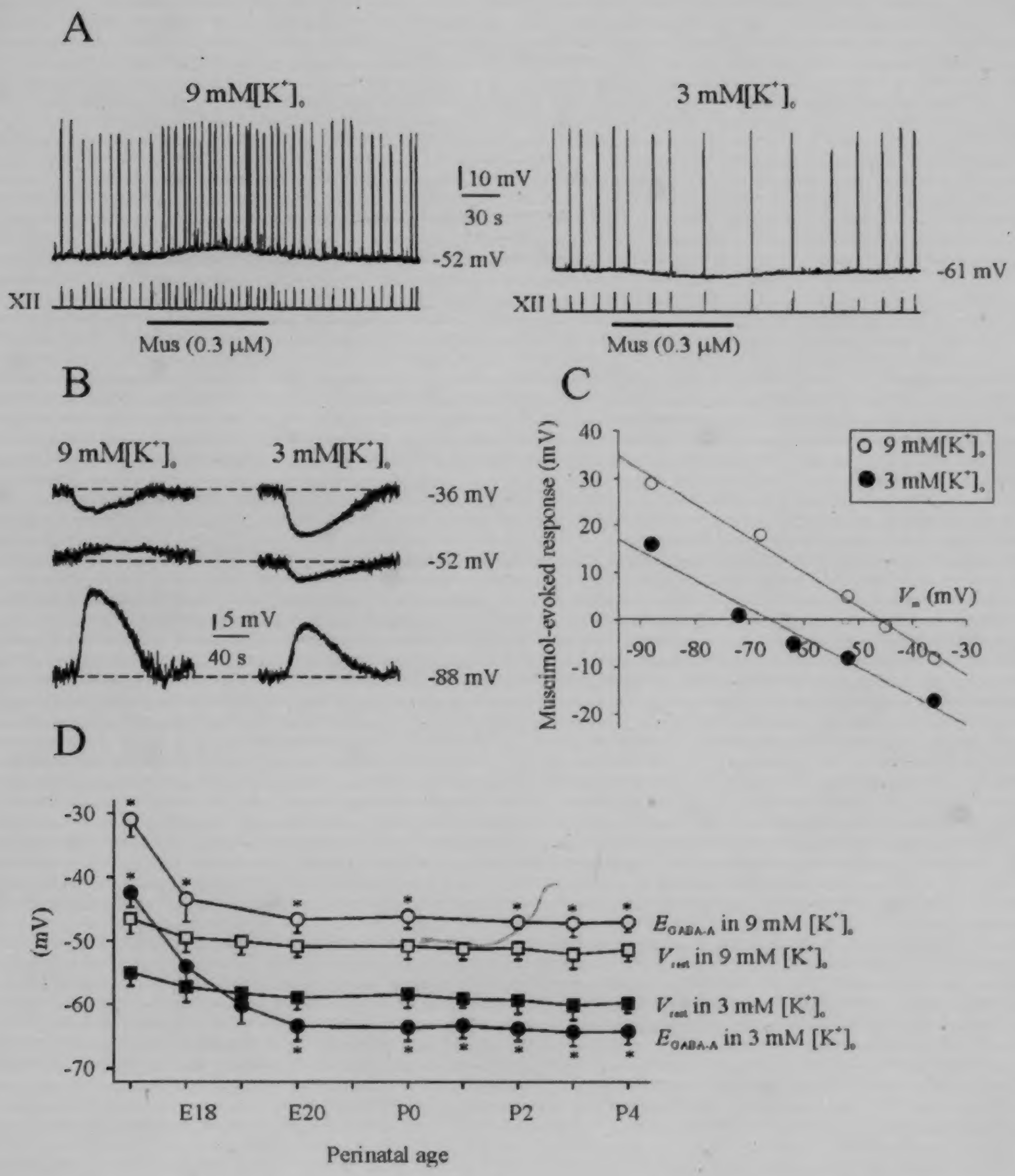

A
9 mM[K+]o
3 mM[K+]o
10 mV
30 s
-52 mV
-61 mV
XII
XII
Mus (0.3 μM)
Mus (0.3 μM)
B
9 mM[K+]o
3 mM[K+]o
-36 mV
-52 mV
5 mV
40 s
-88 mV
C
Muscimol-evoked response (mV)
○ 9 mM[K+]o
● 3 mM[K+]o
Vm (mV)
40
30
20
10
0
-10
-20
-90
-80
-70
-60
-50
-40
-30
D
(mV)
-30
-40
-50
-60
-70
E18
E20
P0
P2
P4
Perinatal age
EGABA-A in 9 mM [K+]o
Vrest in 9 mM [K+]o
Vrest in 3 mM [K+]o
EGABA-A in 3 mM [K+]o

Figure 2.5 Influence of $[K^+]_o$ on V_{rest} and $E_{GABA\text{-}A}$. A) Top traces show perforated-patch recordings from an I neuron in a P2 medullary slice preparation bathed in 9 mM or 3 mM $[K^+]_o$. Bottom traces are suction electrode recordings of XII nerve activity. In 9 mM $[K^+]_o$, V_{rest} of the I neuron was -52 mV and muscimol application caused a membrane depolarization and increase in respiratory frequency. After changing to 3 mM $[K^+]_o$, V_{rest} was -61 mV and muscimol caused a membrane hyperpolarization and decrease in respiratory frequency. (B) Perforated-patch recordings of an I neuron from a P1 medullary slice preparation in the presence of TTX (0.3 μM). Recordings show the responses to bath applied muscimol while the V_m was changed by injection of DC current. C) Linear regression line based on data shown in B. There was an approximately 17 mV depolarizing shift in $E_{GABA\text{-}A}$ when the bathing medium was changed from 3 to 9 mM $[K^+]_o$. D) Summary of V_{rest} and $E_{GABA\text{-}A}$ for all I neurons recorded from at different perinatal ages in medullary slice preparations bathed in 3 or 9 mM $[K^+]_o$. * indicates significant difference between V_{rest} and $E_{GABA\text{-}A}$ (n=4-7; p values <0.05).

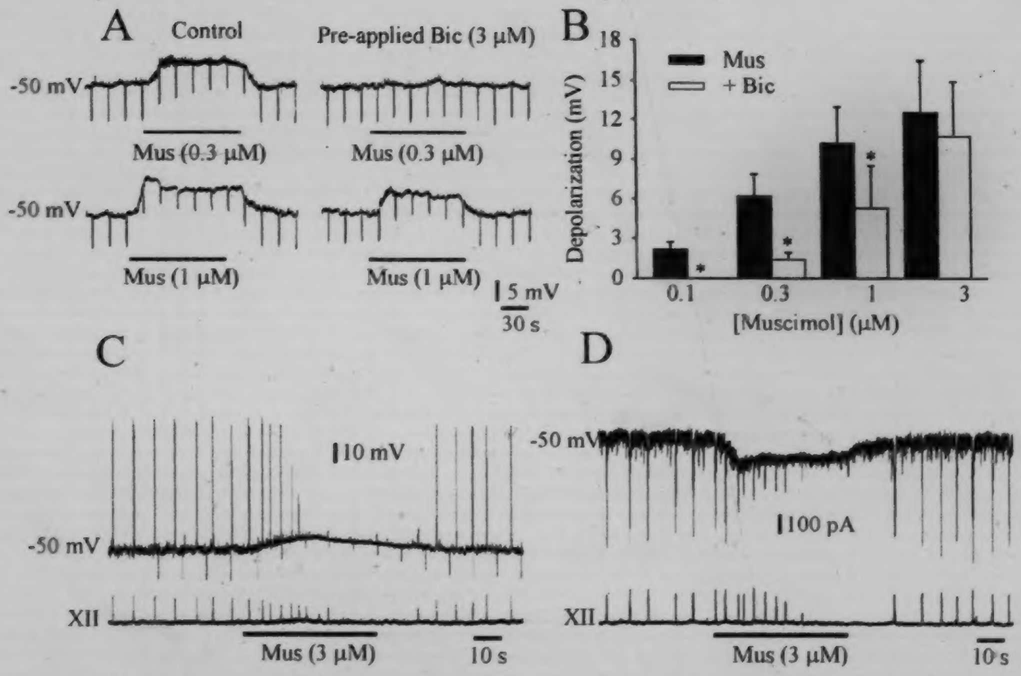


Figure 2.6 Dose-dependant effects of muscimol on V_m and respiratory frequency. A) Perforated-patch recordings of an I neuron from a P1 medullary slice preparation in the presence of TTX (0.3 μM). The input resistance (as determined by application of hyperpolarizing current pulses) of the neuron was decreased and the membrane depolarized by muscimol (left panel). The effects were partially antagonized by bicuculline. B) Population dose-response data showing amount of membrane depolarization in response to muscimol and the effectiveness of bicuculline block. C and D show perforated whole-cell patch recordings of an I neuron in a P1 medullary slice in current (C) and voltage (D) clamp modes (top traces). V_{rest} was -50 mV when bathed in 9 mM $[K^+]_o$. Bottom trace is suction electrode recording of XII nerve activity. Muscimol, at the relatively high concentration of 3 μM, caused a marked depolarization, decrease of the input resistance (C; as determined by application of hyperpolarizing current pulses) and inward current (D) that ultimately resulted in a loss of neuronal spiking. After an initial increase in respiratory frequency, the amplitude of XII motor discharge diminished. *indicates significant difference between muscimol-induced changes in membrane potential with and without bicuculline (p values <0.05).

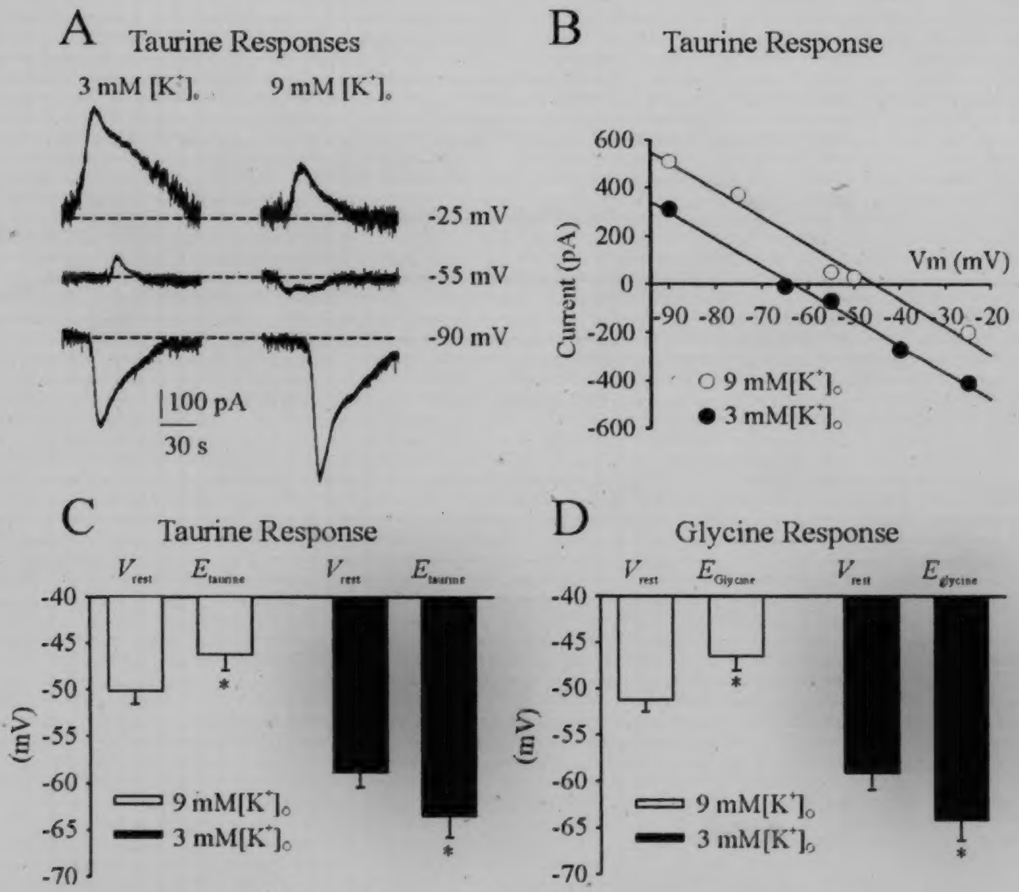


Figure 2.7 Influence of $[K^+]_o$ on V_{rest}, $E_{taurine}$, and $E_{glycine}$. (A) Perforated-patch recordings of an I neuron from a P1 medullary slice preparation in the presence of TTX (0.3 μM). Recordings show the responses to bath applied taurine in the voltage-clamp mode. B) Linear regression line based on data shown in A. C) Summary of V_{rest} and $E_{taurine}$ for all I neurons recorded from P1 medullary slice preparations bathed in 3 or 9 mM $[K^+]_o$. D) Summary of V_{rest} and $E_{glycine}$ for all I neurons recorded from P1 medullary slice preparations bathed in 3 or 9 mM $[K^+]_o$. * indicates significant difference between V_{rest} and $E_{taurine}$ or $E_{glycine}$ (p values <0.05).

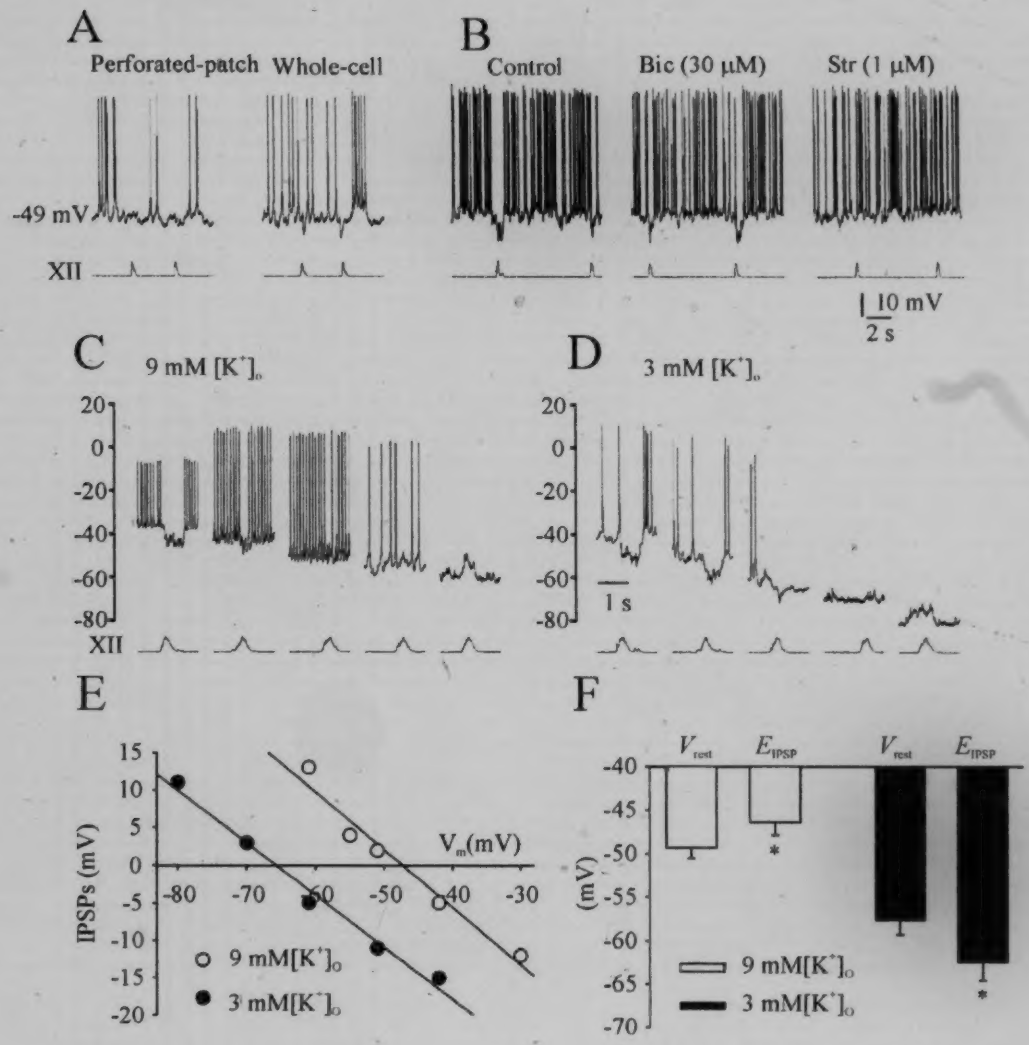

A
Perforated-patch
Whole-cell
-49 mV
XII
B
Control
Bic (30 μM)
Str (1 μM)
10 mV
2 s
C
9 mM [K+]o
20
0
-20
-40
-60
-80
XII
D
3 mM [K+]o
1 s
E
IPSPs (mV)
15
10
5
0
-5
-10
-15
-20
-80
-70
-60
-50
-40
-30
Vm(mV)
9 mM[K+]o
3 mM[K+]o
F
Vrest
EIPSP
(mV)
-40
-45
-50
-55
-60
-65
-70
9 mM[K+]o
3 mM[K+]o
*

Figure 2.8 Characterization of endogenous chloride-mediated inhibition in expiratory neurons. A) Perforated-patch and conventional whole-cell recordings of an E-cell in a P1 medullary slice preparation bathed in 9 mM $[K^+]_o$. V_{rest} was -49 mV with both recording techniques. B) Conventional whole-cell recording from another E neuron in a P1 medullary slice preparation. Strychnine (1μM), but not bicuculline (30 μM) blocked IPSPs during the inspiratory phase. C) Perforated-patch recording of an E neuron in a P1 medullary slice preparation bathed in 9 mM $[K^+]_o$ held at various V_m values. V_{rest} was -51 mV and the E_{IPSP} was between -42 and -51 mV. D) When superfused with 3 mM $[K^+]_o$, V_{rest} was -61 mV was E_{IPSP} between -61 and -70 mV. E) Linear regression line was calculated for the amplitudes of IPSPs at different levels from C and D. F) Summary of V_{rest} and E_{IPSP} for all E neurons recorded from medullary slice preparations bathed in 3 or 9 mM $[K^+]_o$ (each data point is from 4-5 E neurons). * indicates significant difference between V_{rest} and E_{IPSP} (p values <0.05).

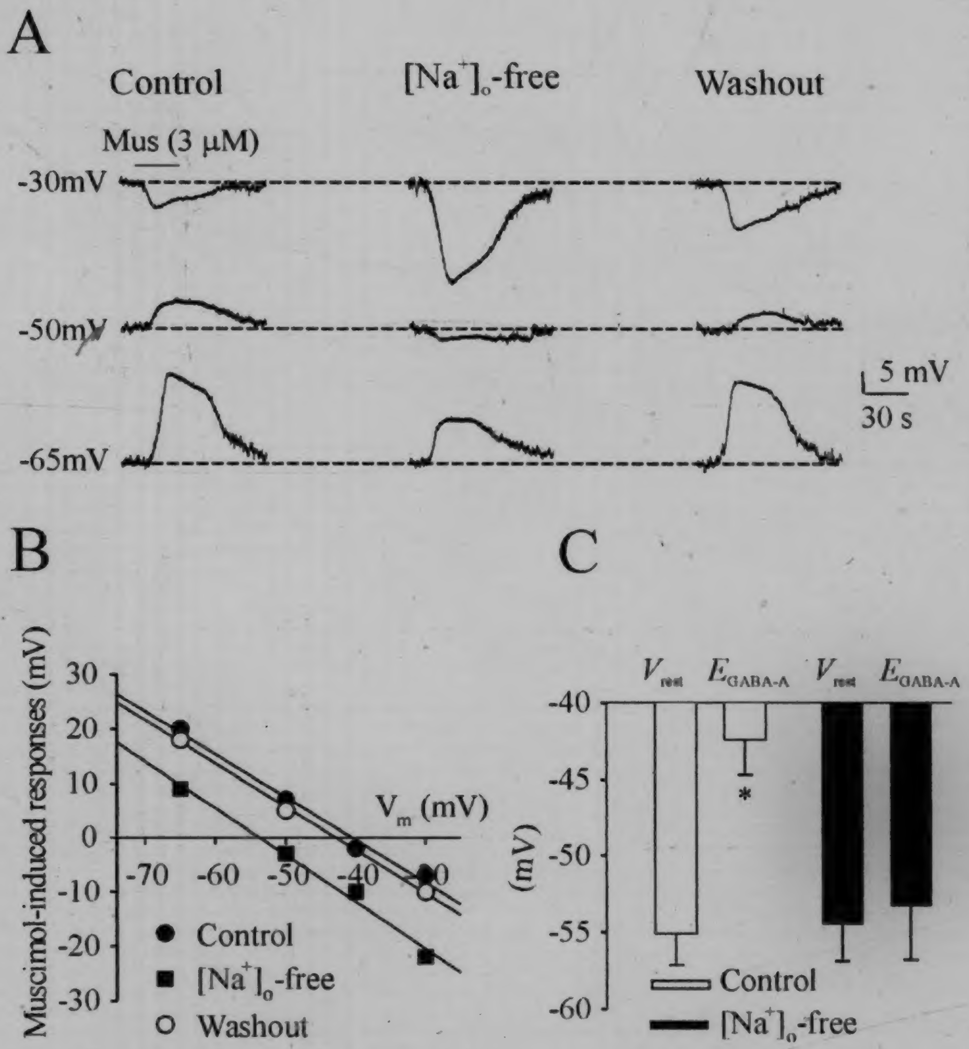
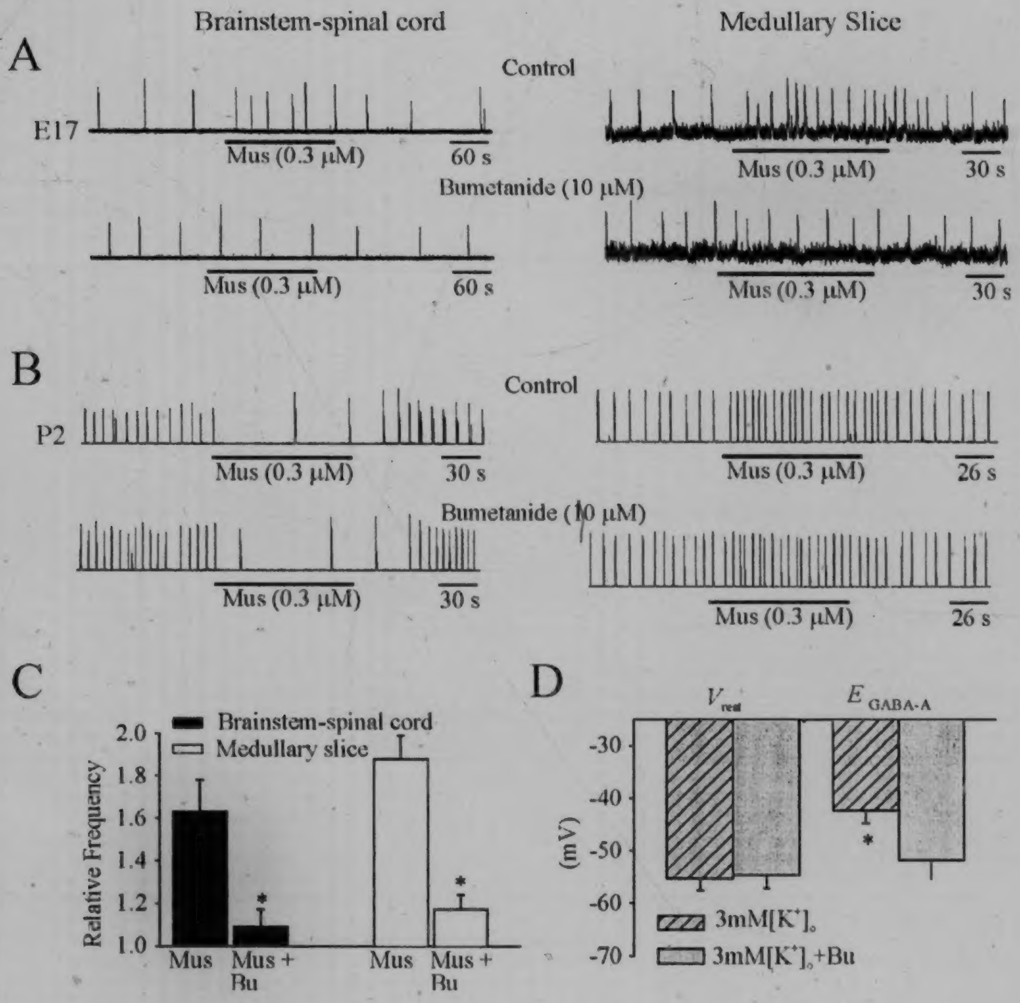


Figure 2.9 Effects of perturbing NKCC1 co-transporter function by removing $[Na^+]_o$. A) Perforated-patch recordings of an I neuron from an E17 medullary slice preparation bathed in 3 mM $[K^+]_o$ in the presence of TTX (0.3 μM). Muscimol was administered to bathing medium containing either control levels of $[Na^+]_o$ or zero $[Na^+]_o$ (replaced with choline) while V_m was changed by injection of DC current. Perturbation of the NKCC1 pump by $[Na^+]_o$ shifted $E_{GABA\text{-}A}$ to a more hyperpolarized value. B) Linear regression line from recordings in A. C) Summary of V_{rest} and $E_{GABA\text{-}A}$ for all neurons recorded from E17 medullary slice preparations bathed in control or zero $[Na^+]_o$. * indicates significant difference between V_{rest} and $E_{GABA\text{-}A}$ (p values <0.05).



Brainstem-spinal cord
Medullary Slice
A
Control
E17
Mus (0.3 μM)
60 s
Mus (0.3 μM)
30 s
Bumetanide (10 μM)
Mus (0.3 μM)
60 s
Mus (0.3 μM)
30 s
B
Control
P2
Mus (0.3 μM)
30 s
Mus (0.3 μM)
26 s
Bumetanide (10 μM)
Mus (0.3 μM)
30 s
Mus (0.3 μM)
26 s
C
Brainstem-spinal cord
Medullary slice
Relative Frequency
2.0
1.8
1.6
1.4
1.2
1.0
*
*
Mus
Mus + Bu
Mus
Mus + Bu
D
V_{rest}
E_{GABA-A}
(mV)
-30
-40
-50
-60
-70
*
3mM[K$^+$]$_o$
3mM[K$^+$]$_o$+Bu

Figure 2.10 Effects of the NKCC1 blocker bumetanide. A) Rectified and integrated suction electrode recordings of C4 ventral roots (brainstem-spinal cord) and XII nerve roots (medullary slice) at E17. Muscimol was added to preparations bathed in control solution and after 30 minutes of bumetanide application. In the presence of NKCC1 blocker, the increase in respiratory frequency caused by muscimol was blocked. B) Similar recording and experimental paradigm as in (A) but at age P2. The responses of both types of *in vitro* preparations were not significantly affected by bumetanide. C) Population data for brainstem-spinal cord and medullary slice preparations showing changes in frequency of respiration relative to control in response to muscimol or muscimol plus bumetanide administration. D) Summary of V_{rest} and $E_{GABA\text{-}A}$ data from gramicidin perforated-patch recordings of I neurons in medullary slices (E17) in 3 mM $[K^+]_o$ in the presence of TTX (0.3 μM), with and without bumetanide (Bu). V_{rest} was not affected by bumetanide but $E_{GABA\text{-}A}$ was shifted to more hyperpolarizing values in E17 *in vitro* preparations. * indicates significant difference between C) muscimol-induced changes in breathing frequency with and without bumetanide and D) V_{rest} and $E_{GABA\text{-}A}$ (p values <0.05).

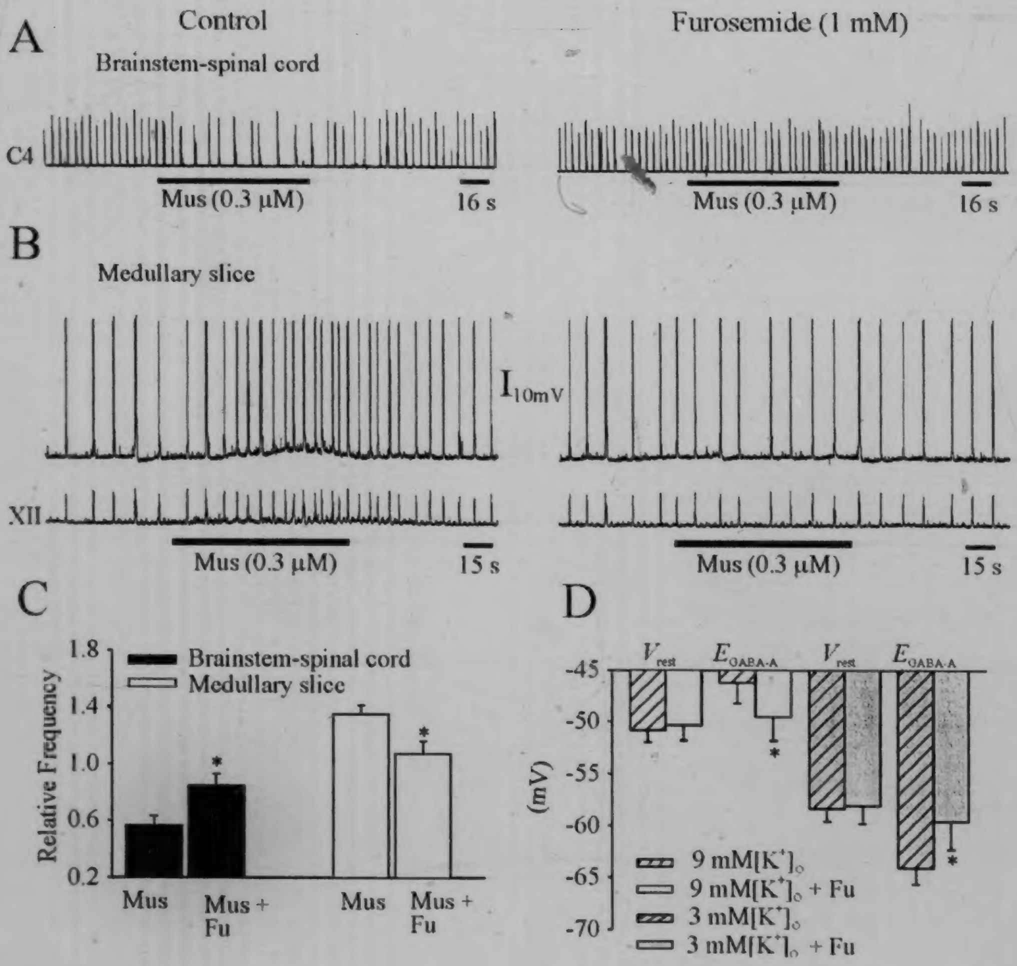

A
Control
Furosemide (1 mM)
Brainstem-spinal cord
C4
Mus (0.3 μM)
16 s
Mus (0.3 μM)
16 s
B
Medullary slice
10mV
XII
Mus (0.3 μM)
15 s
Mus (0.3 μM)
15 s
C
Brainstem-spinal cord
Medullary slice
Relative Frequency
1.8
1.4
1.0
0.6
0.2
*
*
Mus
Mus + Fu
Mus
Mus + Fu
D
V_{rest}
E_{GABA-A}
V_{rest}
E_{GABA-A}
-45
-50
-55
-60
-65
-70
(mV)
*
*
9 mM[K$^+$]$_o$
9 mM[K$^+$]$_o$ + Fu
3 mM[K$^+$]$_o$
3 mM[K$^+$]$_o$ + Fu

Figure 2.11 Effects of the KCC2 blocker furosemide. Rectified and integrated suction electrode recordings of C4 ventral roots in a P1 brainstem-spinal cord preparation. The muscimol-induced decrease of respiratory rhythm (left panel) was diminished after 30 minutes of furosemide application (right panel). B) Perforated-patch recording of an I neuron (top) and XII nerve root recording (bottom) from a P1 medullary slice preparation. The muscimol-induced membrane depolarization and increase of respiratory rhythm (left panel) were diminished in the presence of furosemide (right panel). C) Population data for brainstem-spinal cord and medullary slice preparations showing changes in respiratory frequency relative to control in response to muscimol or muscimol plus furosemide administration. D) Summary of V_{rest} and $E_{GABA\text{-}A}$ data from perforated-patch recordings of I neurons in medullary slices (P1-P2) in 3 or 9 mM $[K^+]_o$, with and without furosemide (Fu). V_{rest} was not affected by furosemide but $E_{GABA\text{-}A}$ was shifted toward V_{rest} in both types of postnatal *in vitro* preparations. * indicates significant difference between C) muscimol-induced changes in breathing frequency with and without furosemide and D) V_{rest} and $E_{GABA\text{-}A}$ (p values <0.05).

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*CHAPTER III

MODULATION OF RESPIRATORY RHYTHMOGENESIS BY NEUROSTEROIDS DURING THE PERINATAL PERIOD

*Previously published paper:

Ren J, Greer JJ (2006) Neurosteroid modulation of respiratory rhythm in rats during the perinatal period. J Physiol 574(2):535-46. Copyright 2006 by the Physiological Society.

3.1 INTRODUCTION

The frequency and amplitude of respiratory rhythmic drive is dynamically regulated to meet the varied demands for ventilation. In particular, rhythmogenesis generated within a key area of the ventrolateral medulla, the preBötzinger complex (preBötC), is modulated by synaptic drive from multiple neurotransmitter systems (reviewed in Rekling & Feldman, 1998). An important inhibitory input arises from GABAergic neurons (Johnson et al., 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998; Ritter & Zhang, 2000). Further, the levels of GABA released are increased during hypoxia, which contribute to hypoxia-induced depression of neonatal ventilation (Huang et al., 1994). In a recent study (Ren & Greer, 2006), we demonstrated the age-dependent changes of $GABA_A$ receptor-mediated actions on respiratory rhythmogenesis during the perinatal period in the rat. Here, we extend upon that work by testing the hypothesis that $GABA_A$ receptor-mediated modulation of perinatal respiration can be profoundly influenced by the presence of neurosteroids.

Steroid hormones and their derivatives regulate neuronal excitability and function (Akwa et al., 1991; Majewska, 1992; Mellon, 1994; Baulieu, 1997; Baulieu & Robel 1996, 1998; Joels, 1997; Jung-Testas & Baulieu, 1998). These actions are mediated by modulating neurotransmitter receptor function, ion channel kinetics and directly via binding to steroid receptors located on neuronal membranes. Neurosteroids within the central nervous system (CNS) arise from two general sources. There are neurosteroids that are synthesized within the CNS from cholesterol or steroid hormone precursors (e.g. pregnenolone, allopregnanolone, progesterone, dehydroepiandorosterone, estradiol, 5α-dihydrotesterone) and those which arrive via the circulation (e.g. testosterone). The level of neurosteroid synthesis in the CNS is particularly high during the perinatal period (Brown & Papdopoulos, 2001; Mellon & Vaudry, 2001) and increases during periods of physiological stress (e.g. hypoxia, parturition, infection; Barbaccia et al., 2001). Neurosteroids, depending on their structure, can act as either negative or positive modulators of $GABA_A$ receptor function (Park-Chung et al, 1999). Thus, there is potential for a neurosteroid-$GABA_A$

receptor interaction with important implications for the control of perinatal breathing. Specifically, the net effect of GABAergic synaptic input to respiratory neuronal populations could vary markedly depending on the types and concentrations of neurosteroids during a given state. In this study, we examined the actions of allopregnanolone and dehydroepiandrosterone sulfate (DHEAS) on the spontaneous respiratory drive generated by *in vitro* models and *in vivo*. These two neurosteroids are prevalent in the perinatal CNS and have pronounced modulatory effects on GABA_A receptor function (Compagnone 1995; Nguyen et al., 2003).

3. 2 MATERIAL AND METHODS

3.2.1 *IN VIVO* AND *IN VITRO* PREPARATIONS AND RECORDINGS

See chapter 2.

3.2.2 PHARMACOLOGICAL AGENTS

All drugs were purchased from Sigma (St. Louis, MO) or RBI (Oakville, ON). The doses of neurosteroids used were based on similar *in vitro* studies and pharmacological profiles of the EC_{50} and IC_{50} (Majewska 1990; Spivak, 1994; Sousa & Ticku, 1997; Rupprecht & Holsboer, 2000). Stock solutions of drugs were prepared as concentrates. All drugs used *in vitro* were dissolved in modified Kreb's solution and the pH adjusted to 7.4. Muscimol (soluble in physiological 0.9% NaCl saline; 0.3-0.5 mg/kg), bicuculline (free base, soluble in DMSO, 0.6-1 mg/kg), allopregnanolone (0.5-4 mg/kg dissolved in DMSO; Gizerian et al., 2004) and DHEAS 10 mg/kg dissolved in DMSO; Hoffman et al., 2003) were administered i.p. *in vivo*.

3.3 RESULTS

3.3.1 MODULATION OF RESPIRATORY FREQUENCY *IN VITRO* BY ALLOPREGNANOLONE

Allopregnanolone had marked effects on respiratory frequency on its own and greatly enhanced the actions of exogenously applied muscimol. Fig 3.1 illustrates the actions of allopregnanolone (1 μM) on respiratory rhythm generated by *in vitro* preparations isolated from P0 rats. The effects of allopregnanolone were time-dependent with a steady-state effect being reached at 10-20 minutes, continuing for 30-60 minutes, which is consistent with previous *in vitro* studies of allopregnanolone actions (Fancsik et al., 2000). The respiratory frequency of brainstem-spinal cord preparations was markedly depressed (34 ± 16% of control, n=6) by allopregnanolone. In contrast, allopregnanolone caused an increase (145 ± 14% of control, n=5) of respiratory frequency generated by medullary slice preparations. The amplitude and duration of inspiratory bursts were not changed significantly. The allopregnanolone effects were antagonized by the $GABA_A$ receptor antagonist bicuculline (3 μM). As demonstrated in Ren & Greer (2006), chloride-mediated conductances are influenced by $[K^+]_o$ due to the role of potassium ions in the function of the KCC2 chloride co-transporter (DeFazio et al., 2000). Thus, the contrasting actions of allopregnanolone in the two types of *in vitro* preparations can be explained by the fact that the brainstem spinal cord and medullary slice preparations are bathed in media containing 3 mM and 9 mM $[K^+]_o$, respectively.

Our focus was on the actions of allopregnanolone and DHEAS. However, we screened a variety of neurosteroid actions *in vitro* and observed that respiratory frequency was modulated by steroid neuromodulators possessing a 3α-hydroxyl group (i.e. allopregnanolone/3α,5α-P, pregnanolone/3α,5β-P, alfaxolone/3α,5α-P, allotetrahydro-corticosterone/3α,5α-P), but not a 3β-hydroxyl group (isopregnanolone/3β,5β-P, pregnenolone/3β-P, 5α-pregnane-3β,20β-diol). Further, the neurosteroids corticosterone, 17β-estradiol, and progesterone had no effects on respiratory frequency at the concentration of 3 μM.

3.3.2 DEVELOPMENTAL CHANGES IN THE ACTIONS OF ALLOPREGNANOLONE *IN VIVO* AND *IN VITRO*

The frequency of breathing in unanesthetized E20 rats *in vivo* was decreased after 10 minutes of allopregnanolone administration (4 mg/kg i.p., Fig 3.2A). The effects of allopregnanolone were prolonged with a steady-state effect being reached at ~30 minutes, continuing for up to one hour. Suppression of breathing was reversed within minutes of the administration of bicuculline (1 mg/kg, i.p., Fig 3.2A). *In vitro*, respiratory frequency was markedly depressed by bath application of allopregnanolone (0.3 μM). The effects of allopregnanolone were prolonged, continuing for more than two hours before recovery (n=4). However, the suppression of respiratory frequency was rapidly reversed by administration of bicuculline (3 μM, Fig 3.2B). The effects of allopregnanolone were dose-dependent with an IC_{50} of 175 ± 4[illegible] Fig 3.2C). Fig 3.2D shows population data for the depression of respiratory frequency *in vitro* and *in vivo* in response to allopregnanolone administration and the reversal of effects by bicuculline (1 mg/kg. i.p. *in vivo*; 3 μM *in vitro*).

We next examined the age-dependent effects of allopregnanolone *in vivo* and *in vitro* (Fig 3.3). Similar to what was observed for the age-dependent effects of chloride-mediated effects (Ren and Greer, 2006), allopregnanolone caused an increase in respiratory frequency in pre-18 brainstem-spinal cord *in vitro* preparations. From E20 onwards, there was a marked depression of respiratory frequency by allopregnanolone. The depression of respiratory frequency was very pronounced at E20-P0. *In vivo*, there was also a marked depression of respiratory frequency during the perinatal period starting at the earliest age studied, E20. Note that due to lung immaturity, it was not feasible to examine foetal rats at earlier stages of development. Administration of 5-10 μl DMSO alone did not affect respiratory frequency.

3.3.3 COMBINATORIAL MODULATORY ACTIONS OF ALLOPREGNANOLONE AND MUSCIMOL

The combinatorial actions of allopregnanolone and muscimol on respiratory frequency were tested using P0 brainstem-spinal cord preparations (Fig 3.4A,B). The respiratory frequency was depressed to 77 ± 15% (n=5) of control by 100 nM muscimol. The respiratory rhythm recovered within five minutes of muscimol washout. Allopregnanolone was then perfused for 20 minutes and, at a concentration of 60 nM, did not cause a significant depression (91 ± 8% of control, n=5) of respiratory frequency. However, after a 20 min pre-application of 60 nM allopregnanolone, the administration of 100 nM muscimol caused a very marked depression of respiratory frequency (34 ± 12% of control, n=5). The respiratory rhythm was not recovered within 20 minutes (n=3), but immediately recovered with application of bicuculline (3 μM; returned to 93 ± 8% of control, n=4).

Plethysmographic recordings were performed to examine the interaction of muscimol and allopregnanolone on regulating respiratory frequency *in vivo* (Fig 3.4C,D). The respiratory frequency was depressed to 81 ± 12% (n=4) of control by administration of low doses of muscimol (i.p., 0.3 mg/kg). The respiratory rhythm recovered within 30 minutes. The respiratory frequency was then depressed to steady-state level (85 ± 10% of control, n=5) 30 minutes after i.p. injection of low doses of allopregnanolone (0.5 mg/kg). The administration of muscimol (i.p., 0.3 mg/kg) caused a further depression of respiratory frequency (41 ± 15% of control, n=4) after 30 min i.p. injection of allopregnanolone (0.5 mg/kg). The respiratory rhythm was not recovered within 1 hour, however, administration of bicuculline (i.p., 0.6 mg/kg) immediately returned the breathing frequency toward control value (90 ± 14% of control, n=4).

Recordings from inspiratory neurons in P0 medullary slices bathed in 3 mM $[K^+]_o$ and TTX (1 μM) were performed to examine the interaction of allopregnanolone and muscimol at the single cell level (Fig 3.5). Note that in the past study (Ren & Greer, 2006) examining chloride-mediated conductances in respiratory neurons, perforated patch recordings were used to avoid perturbation of normal intracellular $[Cl^-]_i$. However, whole-cell patch recordings sufficed for this study directed at examining the interactions of muscimol and neurosteroids. Application of muscimol (100 nM) caused an 3.5 ± 1.3 mV hyperpolarization from V_{rest} of -58 mV and slight decrease (92 ± 7% of control) in membrane resistance (n=7). Administration of 60 nM allopregnanolone on its own (20 minutes) did not cause significant changes in V_m (0.5 ± 1.1 mV hyperpolarization) and membrane resistance (98 ± 6% of control). After a 20 min pre-application of 60 nM allopregnanolone, application of the same dose of muscimol caused an 8.4 ± 3.2 mV hyperpolarization of V_m and a pronounced decrease (58 ± 11% of control, n=6) in membrane resistance. Bicuculline (3 μM, n=5), which did not affect V_{rest} or membrane resistance on its own, antagonized the effects of muscimol plus allopregnanolone.

3.3.4 MODULATION OF RESPIRATORY RHYTHM BY DHEAS

As shown in Fig 3.6A, the inhibition of respiratory rhythm generated by the brainstem- spinal cord preparation caused by muscimol (0.3 μM) was antagonized by DHEAS (30 μM), a negative modulator of $GABA_A$ receptor activation. Similarly, DHEAS antagonized the muscimol-induced stimulation of respiratory rhythm generated by the medullary slice preparation bathed in elevated $[K^+]_o$ (Fig 3.6B). Administration of DHEAS (30 μM) on its own had no significant effects on the frequency of respiratory rhythm in both brainstem-spinal cord and medullary slice preparations. The overall depression of muscimol-induced actions by DHEAS in both types of *in vitro* preparations is plotted in Fig 3.6C. The IC_{50} of 14 ± 3.8 μM is similar to that reported from other studies examining DHEAS-mediated inhibition of muscimol-mediated responses *in vitro* (Majewsak, 1990; Spivak, 1994; Sousa & Ticku, 1997). Application of the related compound DHEA (10-60 μM, n=6) did not modulate muscimol-mediated effects.

Plethysmographic recordings were performed to examine the interaction of muscimol and DHEAS on regulating respiratory frequency *in vivo* (Fig 3.6D,E). Muscimol (0.5 mg/kg) decreased respiratory frequency to 59 ± 15% of control. The muscimol-induced depression of respiratory frequency was reduced to 86 ± 12% after administration of 10 mg/kg DHEAS (n=4).

A further demonstration of the interaction of DHEAS and chloride-mediated conductances via $GABA_A$ receptors is illustrated in Fig 3.7. The endogenous level of

GABA in the brainstem spinal cord preparation was elevated using the GABA-uptake inhibitor nipecotic acid (2 mM for 20 minutes). This caused a marked slowing of respiratory frequency (43 ± 16%) in the brainstem spinal cord preparation. The effects were due to the combination of actions via $GABA_A$ and $GABA_B$ receptors. To minimize the actions via $GABA_B$ receptor, the antagonist to that receptor subtype, saclofen (400 μM), was added to the superfusate. The remaining depression of respiratory frequency was primarily via $GABA_A$ receptor-mediated events (63 ± 12% of control). The further addition of DHEAS (30 μM) returned the respiratory frequency toward control values (86 ± 11% of control). In medullary slice preparations, there was a slight, but statistically insignificant, increase in respiratory frequency after 20 minutes of nipecotic acid application (109 ± 13% of control). However, there was a significant increase (142 ± 11% of control) in respiratory frequency after the administration of saclofen (400 μM). The $GABA_A$ receptor-mediated increase was diminished (115 ± 10% of control) by DHEAS (30 μM).

3.3.5 DEPRESSION OF MUSCIMOL-INDUCED MODULATION OF RESPIRATORY ACTIVITY BY DHEAS

The effects of DHEAS on muscimol-induced outward currents were tested using whole-cell patch recordings of inspiratory neurons under voltage-clamp in P1 medullary slices bathed in 3 mM $[K^+]_o$ and TTX (1 μM) (Fig 3.8). With a holding membrane potential (V_h) of -58 mV, bath application of muscimol (0.3 μM) caused a 78 ± 20 pA outward current (n=5). The muscimol-induced outward current was reduced to 21 ± 14 pA in the presence of bath applied DHEAS (30 μM). DHEAS (30 μM) on its own did not cause any significant current. Reapplication of muscimol after washout of DHEAS induced a 65 ± 22 pA outward current.

3.4 DISCUSSION

Past work has demonstrated that circulating gender hormones progesterone and testosterone stimulate breathing (Behan et al., 2003). Here, we focus on respiratory modulation by neurosteroids synthesized *de novo* in glial and neuronal cells, which begin to express the necessary enzymes for this biochemical process early in development (Mellon & Vaudry, 2001). Neurosteroids interact with the $GABA_A$ receptor and modulate GABAergic activity (Majewska et al., 1986). Data from this study demonstrate that the overall efficacy of $GABA_A$-mediated modulation of respiratory frequency can be markedly regulated by their presence. Specifically, allopregnanolone and DHEAS are positive and negative modulators of the $GABA_A$ receptor function on respiratory neurons, respectively, during the perinatal period.

In a related study, we determined the actions of chloride-mediated conductances on respiratory rhythmogenesis in perinatal rats from the time of inception of fetal inspiratory drive through to the newborn period (Ren & Greer, 2006). The transition from excitatory to inhibitory effects on respiratory rhythmogenesis occurs at approximately E19. By birth, GABA induces a hyperpolarization of the membrane potential in respiratory medullary neurons and a suppression of respiratory frequency. The age-dependant change in the actions of chloride-mediated conductances is regulated by the development of chloride co-transporters (KCC2 and NKCC1).

Allopregnanolone potentiates the actions of GABA and, at higher concentrations, directly gates the $GABA_A$ receptor chloride channel at a site distinct from the GABA binding site (Callachan et al., 1987; Majewska, 1992; Lambert et al., 1996; Ueno et al., 1997; Rupprecht & Holsboer, 2000). The excitability of respiratory neurons in ventrolateral medulla of perinatal rodents is clearly affected by allopregnanolone on its own, and at more physiological levels in the nanomolar range, there was a clear accentuation of muscimol-induced currents. Data from brainstem-spinal cord preparations show a transition from excitatory to inhibitory action of allopregnanolone at E19. This is similar to the transition observed for the effects of the $GABA_A$ receptor agonist muscimol (Ren & Greer, 2006). The post-E19 suppression of respiratory frequency by allopregnanolone was particularly pronounced from E20-P0; also similar to what was observed for muscimol-induced actions. This may reflect developmental changes in $GABA_A$ receptor subunit composition, density and/or distribution within the somata and dendritic membranes. The suppression of respiratory frequency by allopregnanolone was also observed *in vivo*. In all experimental models, the allopregnanolone-mediated actions were countered by the $GABA_A$ receptor antagonist bicuculline. The seemingly paradoxical effects of increased respiratory rhythm by allopregnanolone in the medullary slice preparation can be accounted for by elevated level of $[K^+]_o$ in the media used to bathe the preparation (see Ren & Greer, 2006). Specifically, $[K^+]_o$ influences the function of co-transporters that determine transmembrane Cl^- gradients and thus the net effect of chloride-mediated conductances through the $GABA_A$ receptor.

The physiological relevance of allopregnanolone actions for the control of respiration during the perinatal period is significant. The levels of allopregnanolone and steroidogenic enzymes are particularly high in the brain during the perinatal period (Brown & Papadopoulos, 2001; Mellon & Vaudry, 2001). They are markedly increased further by a variety of stressors including hypoxia, asphyxia, parturition, ethanol exposure and infection; possibly as a neuroprotectant response to prevent excessive excitation (Morrow et al., 1999; Brown & Papadopoulos, 2001; Billiards et al., 2002; Nguyen et al., 2003, 2004). This raises the distinct possibility that neurosteroids depress, in a state-dependent manner, respiratory drive in perinatal mammals. For example, an elevation of allopregnanolone and GABA (Hoop at al., 1999) levels during hypoxia and their combinatorial actions could lead to a profound depression of respiratory drive. Precedent for neurosteroid modulation of respiration comes from a previous study demonstrating the suppression of FBM in sheep by infusion of pregnanolone (Nicol et al., 1999).

DHEAS belongs to the class of neurosteroids that acts to depress the effects of $GABA_A$ receptor ligands. Further it may play a role in neurodevelopment, due to a transient expression of its synthesizing enzyme (Compagnone et al., 2000) and the potential ability of DHEAS to aid in neuronal pathway formation (Compagnone & Mellon, 2000). Data from this study indicate that DHEAS suppressed the inhibition of respiratory frequency caused by $GABA_A$ receptor activation either by muscimol or accentuating the endogenous levels of GABA. It should be noted that DHEAS can also act as a positive modulator of sigma1 and NMDA receptors (Monnet & Maurice, 2006); the implications for those actions on respiratory modulation were not investigated in this study.

In summary, these data should be considered in context with those from a recent study demonstrating the actions of chloride-mediated conductances, including those via $GABA_A$ receptors, on respiratory frequency during the perinatal period (Ren & Greer, 2006). Specifically, the net effect of GABA acting via $GABA_A$ receptors will be determined by the overall balance of negative and positive neurosteroid modulators within respiratory nuclei. This adds a level of complexity that must be considered when examining depression of breathing in mammals associated with various behavioral states and pathogenic conditions such as apnoeas and sudden deaths suspected to be associated with central respiratory dysfunction.

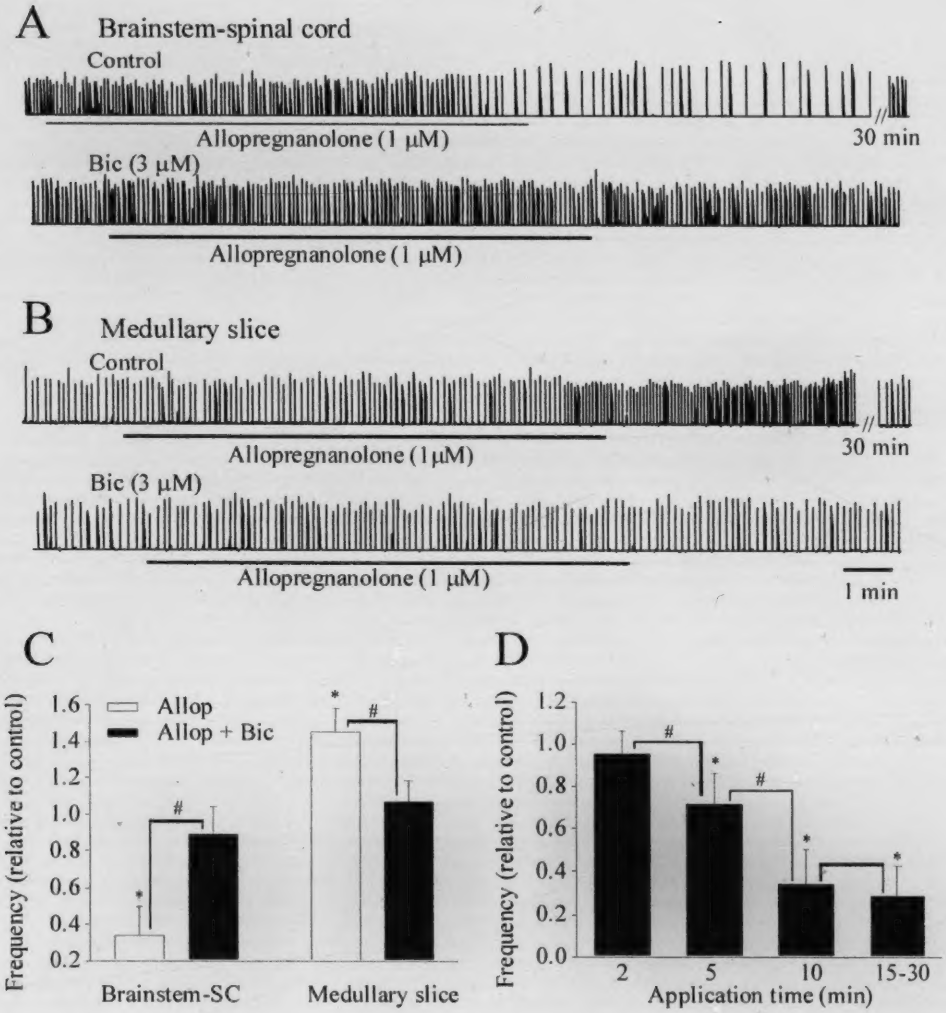
A
Brainstem-spinal cord
Control
Allopregnanolone (1 μM)
30 min
Bic (3 μM)
Allopregnanolone (1 μM)
B
Medullary slice
Control
Allopregnanolone (1 μM)
30 min
Bic (3 μM)
Allopregnanolone (1 μM)
1 min
C
Frequency (relative to control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Allop
Allop + Bic
*
#
Brainstem-SC
Medullary slice
D
Frequency (relative to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
5
10
15-30
Application time (min)

Figure 3.1 Modulation of respiratory frequency by allopregnanolone. A) Rectified and integrated suction electrode recordings of C4 ventral root activity in a P0 brainstem-spinal cord preparation bathed in 3 mM $[K^+]_o$ showing allopregnanolone-induced depression of respiratory frequency. The effects of allopregnanolone (1 μM applied for 10 minutes indicated by solid line) started at approximately 8 minutes, reached to a steady-state maximal level at 15 minutes, continuing for 30 minutes. The effect of allopregnanolone was antagonized by the presence of bicuculline (3 μM). B) Rectified and integrated recordings of XII cranial root activity in medullary slice preparation from a P0 rat bathed in 9 mM $[K^+]_o$ showing allopregnanolone-induced increase of respiratory frequency. Similar to that observed in the brainstem-spinal cord preparations, the effects of allopregnanolone (1 μM for 10 minutes) started at approximately 8 minutes, reached to a steady-state maximal level at 13 minutes, continuing for 35 minutes. The effects of allopregnanolone were antagonized by the presence of bicuculline (3 μM). C) Population data from brainstem-spinal cord (SC) and medullary slice preparations showing changes in frequency of respiration relative to control in response to allopregnanolone (Allop, 1 μM) in the absence and presence of bicuculline (Bic, 3 μM). Each data point was from 5-6 P0 preparations. D) The depression of respiratory frequency by allopregnanolone (1 μM) was increased with the time of the drug application. The maximal effects were achieved with 10 minutes application of allopregnanolone, since there was no significantly further depressing effects observed with 15-30 minutes. Each data point was from 5-8 P0 brainstem-spinal cord preparations. * indicates significant difference relative to control (*p* values <0.05); # indicates significant difference between groups (*p* values <0.05).

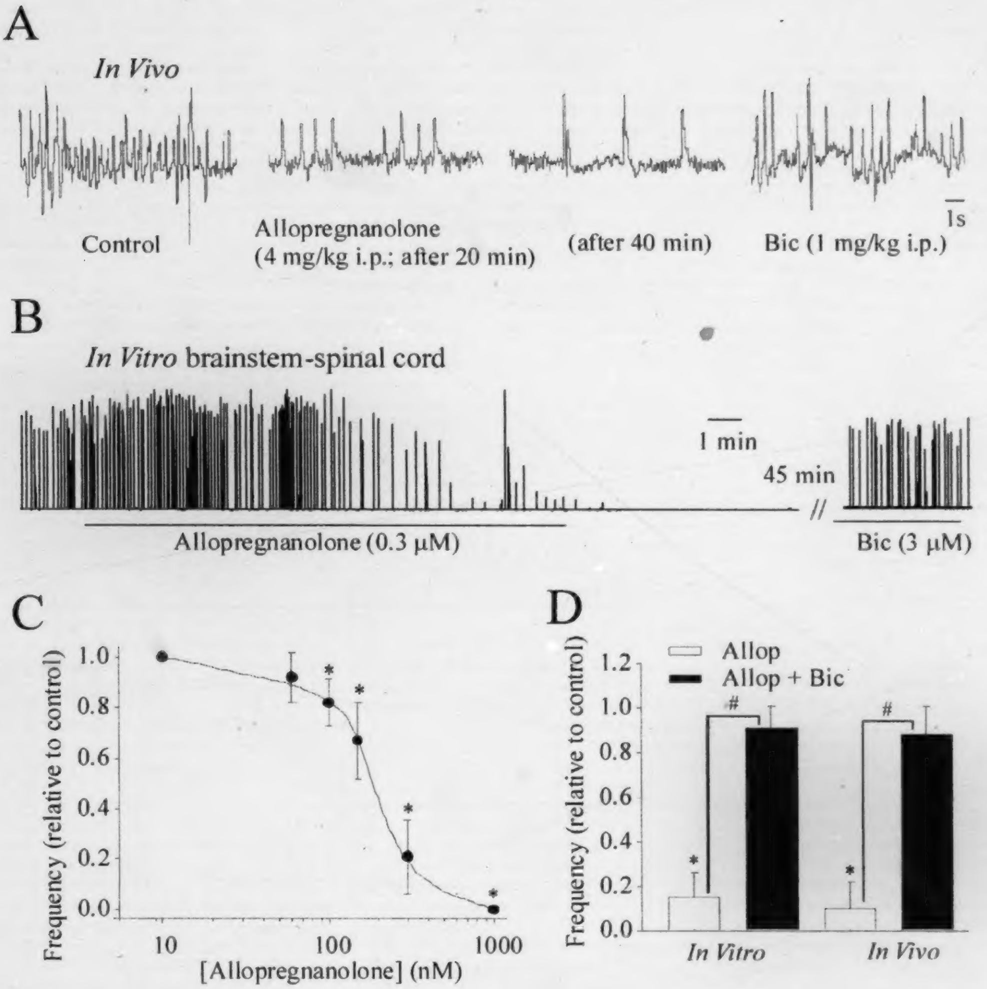
A
In Vivo
Control
Allopregnanolone
(4 mg/kg i.p.; after 20 min)
(after 40 min)
Bic (1 mg/kg i.p.)
1s
B
In Vitro brainstem-spinal cord
1 min
45 min
Allopregnanolone (0.3 μM)
Bic (3 μM)
C
Frequency (relative to control)
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
[Allopregnanolone] (nM)
*
D
Frequency (relative to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Allop
Allop + Bic
#
*
In Vitro
In Vivo

Figure 3.2 Depression of respiratory frequency by allopregnanolone *in vitro* and *in vivo*. A) Whole-body plethysmograph recordings illustrating the allopregnanolone-induced depression of E20 rat breathing frequency. The depression reached a maximum level 40 min after i.p. injection of allopregnanolone and was antagonized by i.p. injection of bicuculline. B) Rectified and integrated recordings of hypoglossal nerve root activity in the brainstem-spinal cord preparation from an E20 rat. The depression reached a maximum level 20 minutes after bath application of allopregnanolone (0.3 μM for 15 minutes). The respiratory rhythm stopped for approximately 1 hour until administration of bicuculline (3 μM). C) Dose-response curve for changes in respiratory frequency of brainstem spinal cord preparations in response to 15 minute bath application of allopregnanolone (E20). Each data point was from 4-8 preparations. D) Comparison of effects of allopregnanolone actions *in vitro* and *in vivo* at E20 in the absence and presence of bicuculline. Each data point was from 4 preparations. * indicates significant difference relative to control (*p* values <0.05); # indicates significant difference between groups (*p* values <0.05).

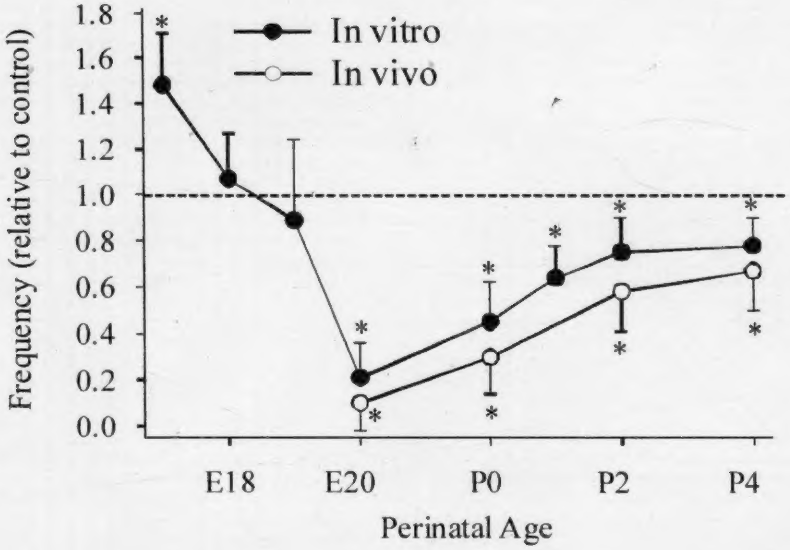

Figure 3.3 Age-depended effects of allopregnanolone *in vitro* (300 nM for 15 min application, E17-P4, black circles) and *in vivo* (4mg/kg, i.p., E20-P4, open circles). Each data point was from 4-8 preparations. * indicates significant difference relative to control (*p* values <0.05).

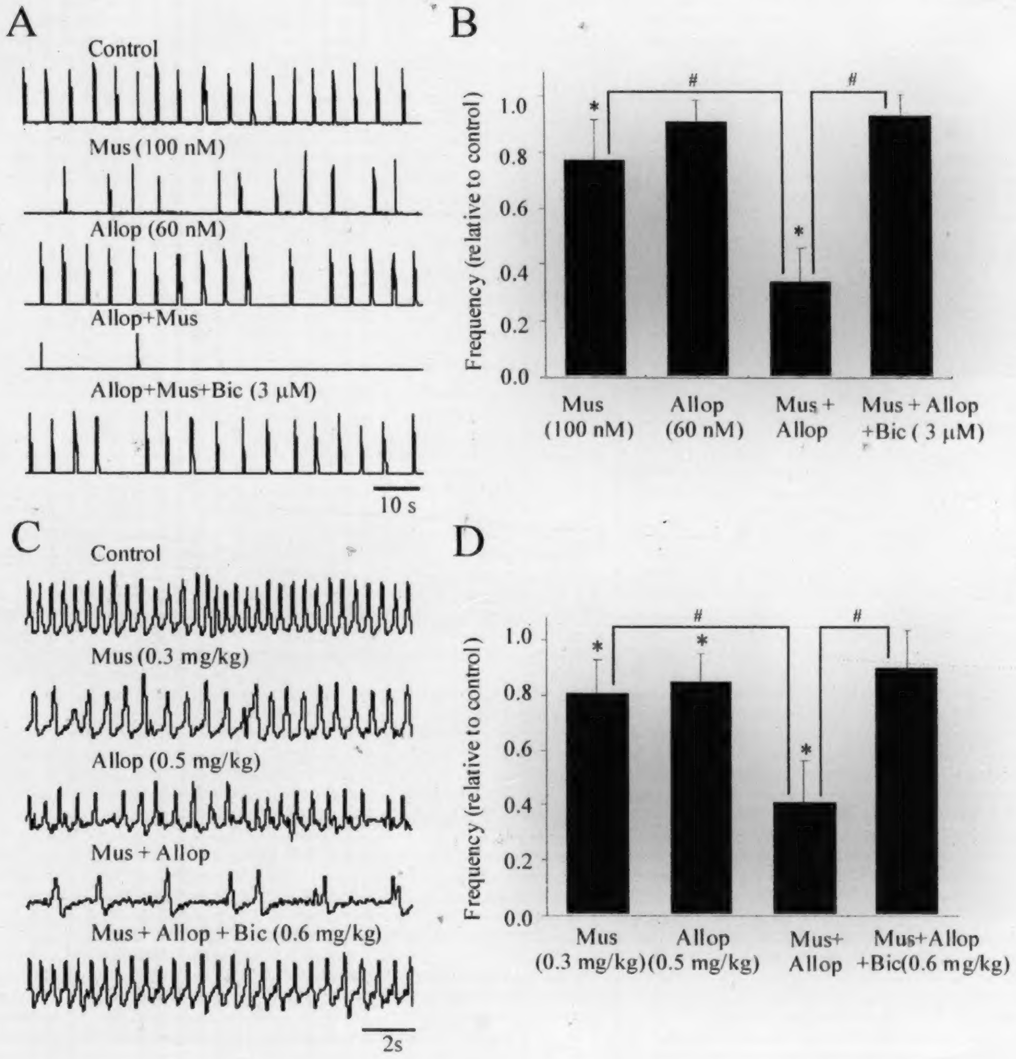
A
Control
Mus (100 nM)
Allop (60 nM)
Allop+Mus
Allop+Mus+Bic (3 μM)
10 s
B
Frequency (relative to control)
1.0
0.8
0.6
0.4
0.2
0.0
*
#
#
*
Mus (100 nM)
Allop (60 nM)
Mus + Allop
Mus + Allop +Bic (3 μM)
C
Control
Mus (0.3 mg/kg)
Allop (0.5 mg/kg)
Mus + Allop
Mus + Allop + Bic (0.6 mg/kg)
2s
D
Frequency (relative to control)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
#
#
*
Mus (0.3 mg/kg)
Allop (0.5 mg/kg)
Mus+ Allop
Mus+Allop +Bic(0.6 mg/kg)

Figure 3.4 The combinatorial action of allopregnanolone and the $GABA_A$ receptor agonist muscimol *in vitro* and *in vivo*. A) Rectified and integrated suction electrode recordings of C4 ventral root activity in brainstem-spinal cord preparation from a P0 rat. Traces show effects of muscimol and allopregnanolone compared to the combined application of both. The very marked depression of respiratory frequency caused by allopregnanolone and muscimol was blocked by bicuculline. B) Population data showing changes in respiratory frequency relative to control for each of the drug protocol administrations shown in A. Each data point was from 4-5 P0 preparations. C) Whole-body plethysmograph recordings from an unanesthetized rat pup showing the effects of i.p. administration of muscimol (0.3 mg/kg) or allopregnanolone (0.5 mg/kg). Either agent caused a small depression of breathing. However, when muscimol (i.p., 0.3 mg/kg) was administered 30 minutes after i.p. injection of allopregnanolone (0.5 mg/kg) (trace 4), a period of apnoea occurred. The depression of respiratory frequency caused by muscimol and allopregnanolone was antagonized by bicuculline (0.6 mg/kg). D) Population data showing changes in respiratory frequency relative to control for each of the drug protocol administrations shown in C. Each data point was from 4 P0 preparations. * indicates significant difference relative to control (*p* values <0.05); # indicates significant difference between groups (*p* values <0.05).

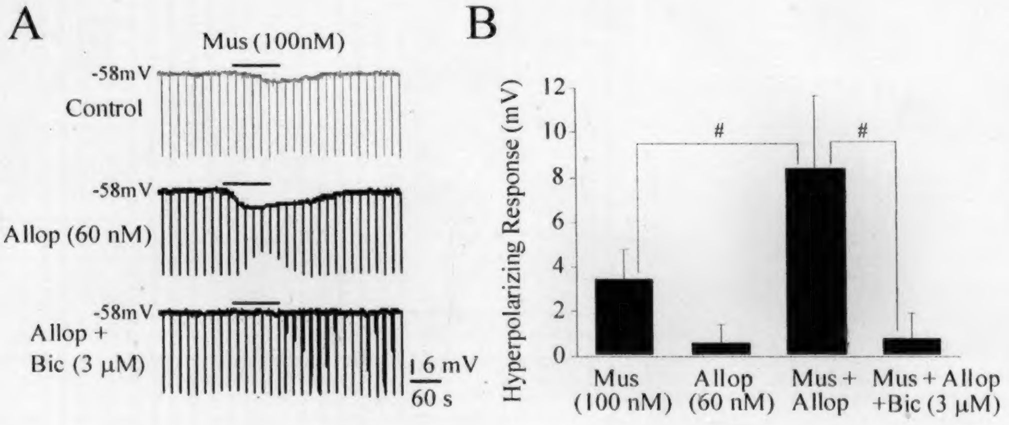

Figure 3.5 Effects of allopregnanolone on muscimol-induced membrane hyperpolarizations. A) Whole-cell patch clamp recording from an inspiratory neuron in a P0 medullary slice preparation bathed in 3 mM $[K^+]_o$ and TTX (1 μM). The muscimol-induced hyperpolarization and decrease in input resistance (determined by application of hyperpolarizing current pulses) were amplified in the presence of allopregnanolone. Effects were antagonized by bicuculline. B) Population data showing hyperpolarizing response for each of the drug applications shown in A. Each data point was from 5-7 P0 preparations. # indicates significant difference between groups (p values <0.05).

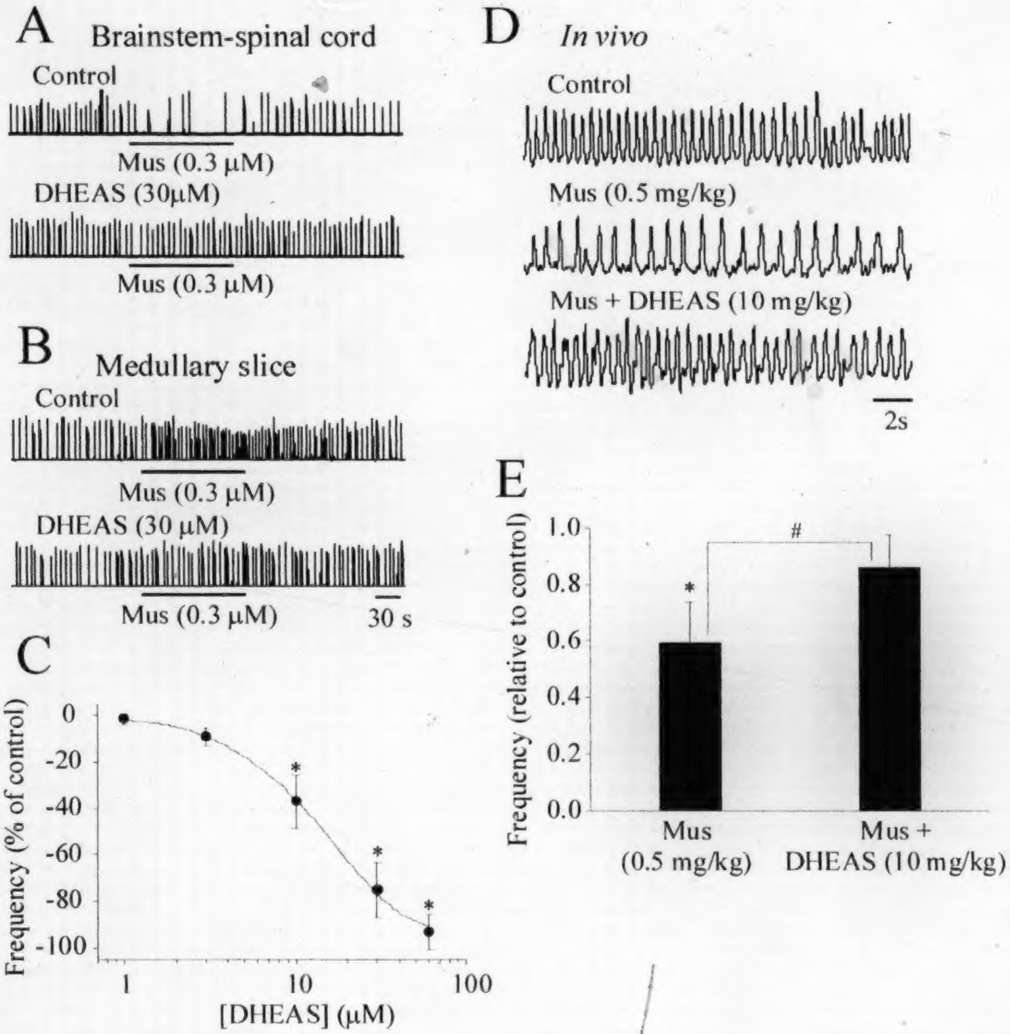
A
Brainstem-spinal cord
Control
Mus (0.3 μM)
DHEAS (30μM)
Mus (0.3 μM)
B
Medullary slice
Control
Mus (0.3 μM)
DHEAS (30 μM)
Mus (0.3 μM)
30 s
C
Frequency (% of control)
0
-20
-40
-60
-80
-100
*
*
*
1
10
100
[DHEAS] (μM)
D
In vivo
Control
Mus (0.5 mg/kg)
Mus + DHEAS (10 mg/kg)
2s
E
Frequency (relative to control)
1.0
0.8
0.6
0.4
0.2
0.0
#
*
Mus
(0.5 mg/kg)
Mus +
DHEAS (10 mg/kg)

Figure 3.6 Effects of DHEAS on muscimol-induced changes of respiratory frequency *in vitro* and in vivo. A) Rectified and integrated suction electrode recordings of C4 ventral root activity in a P0 brainstem-spinal cord preparation. The inhibition of respiratory frequency by muscimol was antagonized by DHEAS. B) Rectified and integrated suction electrode recordings of XII nerve roots in P0 medullary slice preparation. The muscimol-induced increase of respiratory frequency in 9 mM $[K^+]_o$ bathing solution was antagonized by DHEAS. C) Dose-response curve for the DHEAS blockade of muscimol-induced effects of respiratory frequency in brainstem-spinal cord and medullary slice preparations. Each data point was from 5-8 P0 preparations. D) The effects of i.p. administration of muscimol (0.5 mg/kg) to an unanesthetized P0 rat pup. The muscimol-induced depression of breathing was antagonized by DHEAS (10 mg/kg). E) Population data showing changes in respiratory frequency relative to control for each of the drug protocol administrations shown in D. Each data point was from 4 P0 preparations. * indicates significant difference relative to control (p values <0.05); # indicates significant difference between groups (p values <0.05).

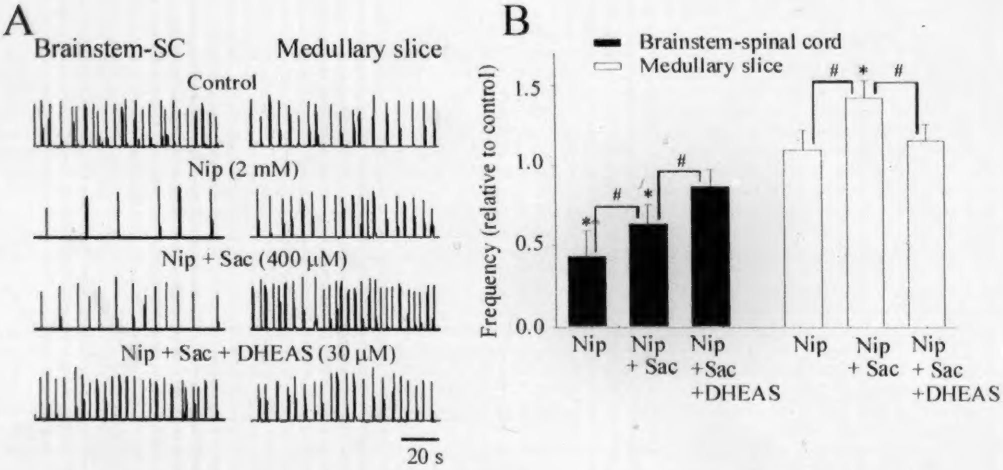

Figure 3.7 The effects of DHEAS on the changes of respiratory frequency caused by elevated levels of endogenous GABA. A) Rectified and integrated suction electrode recordings of C4 ventral root in P0 brainstem-spinal cord (left panel) and XII nerve root activity in P0 medullary slice (right panel) preparations. Endogenous levels of GABA were elevated by bath application of the GABA uptake inhibitor nipecotic acid (Nip). This resulted in a clear decrease in respiratory frequency of brainstem-SC preparations and a slight, but insignificant increase in medullary slice preparations after 20 minutes of nipecotic acid application. The antagonist to $GABA_B$ receptor, saclofen (Sac), was added to the bathing medium to minimize the component of the respiratory frequency depression resulting from the actions of $GABA_B$ receptors. The remaining $GABA_A$ receptors mediated decrease and increase of respiratory frequency in brainstem-spinal cord and medullary slice preparations, respectively, was depressed by DHEAS. B) Population data showing changes in respiratory frequency relative to control for *in vitro* preparations exposed to the drug paradigms shown in A. Each data point was from 4 P0 preparations. * indicates significant difference relative to control (*p* values <0.05); # indicates significant difference between groups (*p* values <0.05).

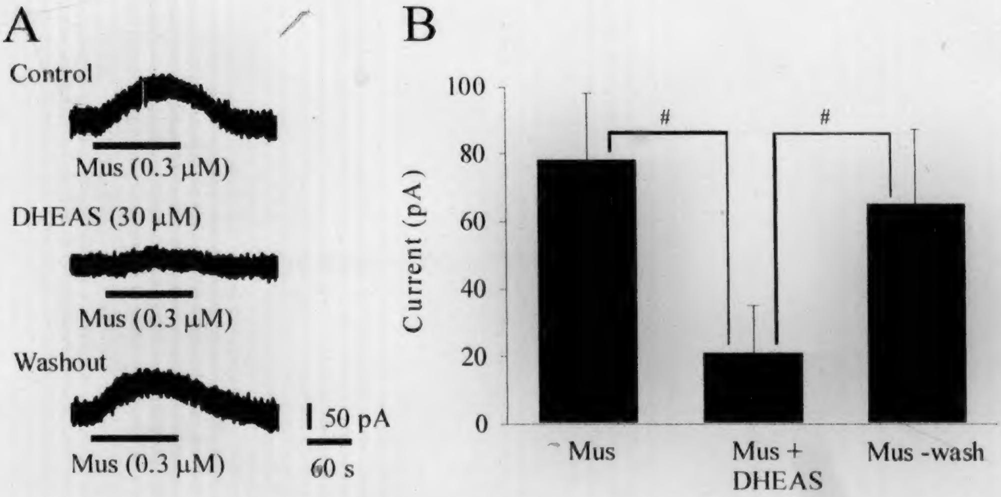

Figure 3.8 Effects of DHEAS on muscimol-induced outward currents. A) Whole-cell patch clamp recording from an inspiratory neuron in a P1 medullary slice preparation bathed in 3 mM $[K^+]_o$ and TTX (1 μM). The neuron was clamped at -58 mV in voltage-clamp mode and the outward current caused by bath application of muscimol recorded. The muscimol-induced current was depressed by bath application of DHEAS (30 μM) and recovered after 5 minutes washout of DHEAS. B) Population data showing the amplitude of outward current measured in response to muscimol and muscimol plus DHEAS. Each data point was from 5 P1 preparations. # indicates significant difference between groups (*p* values <0.05).

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*CHAPTER IV

ABSENCE OF NECDIN, ENCODING THE PRADER-WILLI SYNDROME-DELETED GENE *NECDIN*, RESULTS IN CONGENITAL DEFICIENCY OF CENTRAL RESPIRATORY DRIVE IN NEONATAL MICE

*Previously published papers:

1. Ren J#, Lee S#, Pagliardini S, Gérard M, Stewart CL, Greer JJ, Wevrick R (2003) Absence of Ndn, encoding the Prader-Willi syndrome-deleted gene *necdin*, results in congenital deficiency of central respiratory drive in neonatal mice. J Neurosci 23(5):1569-73. Copyright 2003 by the Society for Neuroscience. #These authors contributed equally.

2. Pagliardini S, Ren J, Wevrick R, Greer JJ (2005) Developmental abnormalities of neuronal structure and function in prenatal mice lacking the Prader-Willi syndrome gene necdin. Am J Pathol 167:175-91. Copyright 2005 by American Society for Investigative Pathology.

My contribution to this project consisted in the planning and execution of the functional and electrophysiological study. The anatomical study was performed by Drs. S. Lee & S. Pagliardini.

4.1 INTRODUCTION

Necdin (neurally differentiated EC-cell derived factor) is one of four known protein coding genes that are deficient in people with Prader-Willi syndrome (PWS) (Jay et al., 1997; MacDonald & Wevrick, 1997; Sutcliffe et al., 1997). PWS is a developmental neurobehavioral disorder (Online Mendelian Inheritance in Man entry #176270) that occurs sporadically at a frequency of about 1 in 15,000 (Holm et al., 1993). The major manifestations of PWS include neonatal hypotonia and failure to thrive, followed by childhood-onset developmental delay and obesity. Infants with PWS have significant respiratory abnormalities including sleep-related central and obstructive apneas and reduced response to changes in oxygen and CO_2 levels (Arens et al., 1994; Gozal et al., 1994; Clift et al., 1994; Wharton & Loechner, 1996; Shluter et al., 1997; Menendez, 1999; Manni et al., 2001; Nixon & Brouillete, 2002). A subset of genes in the region deleted in PWS, including the *NDN* gene encoding necdin, are active only on the paternally inherited allele and silenced by imprinting on the maternal allele (Nicholls, 2000). The relative contribution of the loss of each gene to the complex PWS phenotype is as yet unknown, and there are no known cases of PWS due to deficiency of only one protein-encoding gene.

Necdin was originally identified as a gene up-regulated during the retinoic-acid induced differentiation of P19 embryonic carcinoma cells into neurons (Maruyama et al., 1991). The expression of necdin in mouse development mirrors the cultured cell system, as necdin is expressed in many but not all post-differentiation stage neurons. *Necdin* is a member of the MAGE/necdin gene family that also includes *MAGEL2,* also deficient in PWS (Boccaccio et al., 1999; Lee et al., 2000).

Three necdin-deficient mouse strains were independently generated by homologous recombination in ES cells (Gerard et al., 1999; Muscatelli et al., 2000; Tsai et al., 1999). In all three strains, heterozygous mice that inherit the mutated allele maternally are indistinguishable from their wild type littermates, because of imprinting that normally silences the maternal allele. Two *necdin*-deficient mouse strains carrying a paternally inherited *Ndn* deletion allele are affected by postnatal lethality. Deficiency of *necdin* in these mice causes neonatal respiratory distress that is usually fatal, and surviving mice exhibit mildly abnormal behavior (Gerard et al., 1999; Muscatelli et al., 2000). In the original targeted allele of Gérard *et al.*, there is approximately 70% lethality in the first 30 postnatal hours. Deletion of the PGK-neo cassette present in the original targeted allele increased the lethality to 98% in the *Ndn*^{tm2Stw} necdin-deficient strain (Gerard et al., 1999), possibly because of an effect on nearby genes of the neomycin promoter.

Functional defects of the lungs, respiratory musculature, chemoreception or central neural control mechanisms could account for the respiratory distress phenotype. In this study, we used *in vitro* preparations to assess the respiratory neuronal activity at multiple sites along the central neuraxis. Specifically, we test the hypothesis that the hypoventilation results from a defective central respiratory drive in *necdin*-deficient mice.

4.2 MATERIAL AND METHODS

4.2.1 MOUSE BREEDING AND GENOTYPING

Procedures for animal care were approved by the Animal Welfare Committee at the University of Alberta. *Ndn*^{tm2Stw} *necdin*-deficient mice were bred through the maternal line with C57BL/6J male mice. Male offspring carrying a maternally inherited *Ndn*^{tm2Stw} are phenotypically normal, and were bred to C57BL/6J females to produce experimental embryos and offspring. In these litters, half the mice are wild-type, and half carry a paternally inherited *necdin* deficiency and are functionally null. The timing of pregnancies was determined from the appearance of sperm plugs in the breeding cages (E0.5). Identification of mutant offspring was carried out by PCR genotyping with LacZ oligonucleotide primers (LACZ1942F, 5'GTGTCGTTGCTGCATAAACC & LACZ2406R, 5'TCGTCTGCTCATCCATGACC) or by histochemical detection of spare tissue. For detection of β-galactosidase activity, tissue samples were fixed in cold 0.5%paraformaldehyde and 2.5% gluteraldehyde in 0.1 M phosphate buffer, pH 8. The samples were incubated in β-galactosidase stain until appropriate stain intensity was observed.

4.2.2 *IN VITRO* PREPARATIONS AND ELECTROPHYSIOLOGICAL RECORDINGS

See chapter 2.

4.2.3 RNA *IN SITU* HYBRIDIZATION

A cloned PCR product containing partial open reading frame and 3' untranslated region of mouse *Ndn* (base positions 162-1235 in accession GenBank #M80840) was used as a template for riboprobe synthesis. The digoxygenin labeled RNA antisense riboprobe was synthesized using T7 RNA polymerase and DIG RNA labeling kit (Roche Biochemicals). Cryostat sections, 60 μm thick, were processed for *in situ* hybridization essentially as described (Wilkinson and Nieto, 1993). Processed sections were hybridized on slides at 68°C overnight. Post hybridization washes were at 68°C with no ribonuclease A treatment. Levamisole (2mM) was added to all subsequent steps. Slides were preblocked with 5% blocking reagent (Roche Biochemicals) prior to incubation with preabsorbed antibodies for 6 hours at room temperature.

4.3 RESULTS

4.3.1 RESPIRATORY RHYTHM ARE PERTURBED IN *Ndn*^{tm2Stw} MUTANT NEWBORN MICE

In litters of newborn mice born to a heterozygous *Ndn*^{tm2Stw} male and wild-type female, we observed that a subset of pups gasped for air, turned cyanotic and died over a post-natal time course of a few hours, as previously noted (Gerard et al., 1999; Muscatelli et al., 2000). Nudging the pups caused a transient increase in respiration and loss of cyanosis. Pups exhibiting normal (n=7) and abnormal (n=8) respiration were selected and brainstem-spinal cord preparations isolated within 20 minutes of birth. Amongst pups with abnormal breathing patterns *in vivo,* 7 of 8 failed to generate rhythmic motor bursts from cervical or hypoglossal nerve roots *in vitro*. The remaining pup generated a severely irregular rhythmic motor output. Subsequent genotyping confirmed that preparations with markedly perturbed respiratory rhythms were *Ndn*^{tm2Stw} mutants. While these data were informative, the fact that newborns with respiratory dysfunction were hypoxic and stressed during the early postnatal period could have been a confounding factor. For instance, the central neural control mechanisms could have been compromised secondarily to a primary defect of lung function or peripheral respiratory afferent input. Therefore, we proceeded to assess the central drive in embryos delivered via caesarean section at E18.5.

4.3.2 RESPIRATORY DISCHARGE IN *Ndn*^{tm2Stw} MUTANT EMBRYOS AT E18.5

Simultaneous suction electrode recordings of inspiratory motor discharge were made from diaphragm muscle and/or hypoglossal (XII) nerve roots in brainstem-spinal cord preparations with the ribcage and diaphragm attached. A total of 36 putative necdin-deficient (abnormal respiratory rhythm) and 22 wild-type E18.5 embryonic mice were subsequently selected for detailed analyses. In each case, post-experimental genotyping confirmed the identity of wild type and *Ndn*^{tm2Stw} mutant mice. In *Ndn*^{tm2Stw} mutant mice, the rhythms were consistently irregular with prominent bouts of respiratory depression characterized by burst frequencies of 1-3 bursts per 10-minute period and central apneas persisting for up to several minutes (Fig 4.1A). The bouts of suppressed respiratory rhythmic discharge were interspersed with periods of inspiratory motor bursts close to frequencies observed in wild-type preparations (Table 4.1). There were no marked differences in the amplitude or duration of inspiratory bursts. These recordings demonstrate that the defect in rhythmic motor discharge is present in both cranial and spinal motoneuron populations.

We selected 18 of the *Ndn*^{tm2Stw} mutant mouse preparations and removed the ribcage and diaphragm musculature. The rhythmic discharge pattern recorded from the fourth cervical root was similar to that recorded from the diaphragm EMG in 7 of 18 preparations. The other 11 *Ndn*^{tm2Stw} mutant mice failed to produce any respiratory motor output from cervical or hypoglossal nerve roots upon removal of the ribcage and diaphragm musculature. Presumably, the threshold excitation necessary to achieve rhythmic motor output in these mutants was only achieved with the intact musculature and associated afferent input.

4.3.3 MEDULLARY SLICE PREPARATIONS FROM *Ndn*^{tm2Stw} MUTANT EMBRYOS AT E18.5

We recorded rhythmic respiratory discharge from the hypoglossal (XII) motoneuron pool in medullary slice preparations isolated from *Ndn*^{tm2Stw} mutant and wild-type mice (Fig 4.1B). The rhythmic neuronal discharge was irregular in all *Ndn*^{tm2Stw} mutant mice (n=10), while robust and regular in all wild-type (n=8) preparations. There were no cases in which *Ndn*^{tm2Stw} medullary slice preparations failed to generate some sort of rhythmic motor output. The elevated extracellular K^+ (9mM) provided sufficient excitatory drive to respiratory neuronal populations to reach a threshold for generating a rhythmic, albeit irregular, pattern.

We next determined whether or not the abnormal respiratory rhythm was present within the pre-Bötzinger complex. Suction electrode recordings of population neuronal activity were performed in the region of the preBötC. The rhythmic discharge of neurons within the preBötC of mutant preparations had the same abnormal characteristics as the XII motor discharge (Fig 4.1B, Table 4.1). Next, whole-cell patch clamp recordings of inspiratory neurons within the preBötC were performed. As illustrated in Fig 4.2, the neurons fired with an irregular rhythm with prolonged periods of suppressed rhythmogenesis. The resting membrane potential of inspiratory neurons became more depolarized during epochs of increased respiratory rhythmic frequency. There were also bouts of longer duration bursting activity that is not of respiratory origin (Greer et al., 1992).

4.3.4 NECDIN mRNA EXPRESSION IN THE MEDULLA

Previous investigations of *necdin* gene expression by RNA *in situ* hybridization or immunohistochemistry had focused on the cerebrum, cerebellum, and the hypothalamus (Uetsuki et al., 1996; Niinobe et al., 2000). Expression of the *Ndn*^{tm2Stw} LacZ reporter gene had been noted in the medulla, spinal cord and dorsal root ganglia in E17 embryos (Gerard et al., 1999). We examined the expression of necdin by RNA *in situ* hybridization in wild-type medullary sections at E15.5, when respiratory activity commences, and E18.5, the stage used for electrophysiological recordings. This experiment was to determine whether only subpopulations of neurons express necdin, as observed in other structures of the nervous system. Necdin expression was evident in the ventrolateral medulla where the respiratory rhythm generator is located, but levels here were not significantly different than in other medullary regions (Fig 4.3).

4.3.5 NEUROCHEMICAL MODULATION OF RESPIRATORY FREQUENCY OF *Ndn*^{tm2Stw} MUTANT EMBRYOS AT E18.5

Finally, we tested the hypothesis that the respiratory rhythm could be normalized in the presence of exogenously applied neurotransmitter agonists (SP and TRH) known to excite neurons within the preBötC region (Greer et al., 1996; Ptak & Hilaire, 1999). As shown in Fig 4.4, addition of SP (1 μM) and TRH (1 μM) significantly modulated the rhythm generated by Ndn^{tm2Stw} mouse brainstem-spinal cord preparations. The frequency of discharge during the previously slow periods was increased markedly and the incidence of apneas diminished. However, the fluctuations in respiratory frequency between slow and fast rhythmogenesis persisted. The application of SP (1 μM) to medullary slice preparations had similar modulatory effects (data not shown). Further, exogenous application of serotonin (25 μM) or noradrenaline (3-30 μM) resulted in the same excitatory response (data not shown). Thus, we concluded that addition of appropriate neuromodulatory drive to the preBötC region could alleviate the long periods of slow respiratory rhythms and apnea, however, the overall respiratory rhythm instability persisted.

We also tested the actions of growth hormone (GH) due to the fact that it is effective in alleviating apnea in Prader-Willi syndrome infants (Menendez, 1999). The stimulatory effects of GH observed clinically are likely not due to direct stimulation of the preBötC as exogenous application (1-15 nM) did not affect respiratory frequency in either wild-type or mutant *in vitro* preparations. Neither did insulin-like growth factor (IGF-1; 10-40 nM), an intermediate effector of GH action, have any noticeable effect on respiratory neural discharge *in vitro*.

4.4 DISCUSSION

Necdin-deficient *Ndn*^{tm2Stw} newborn mice hypoventilate, rapidly turn cyanotic and die. We sought to assess centrally generated respiratory rhythmogenesis and drive transmission in isolation from other aspects of the respiratory system (e.g., lung function, peripheral afferent feedback). The brainstem-spinal cord-diaphragm preparation has been well characterized and shown to generate a complex, coordinated pattern of respiratory activity (Smith et al., 1990). Recordings of diaphragmatic EMG, cervical ventral roots and hypoglossal roots provide information regarding inspiratory drive transmission to key components of the respiratory motor system. The respiratory motor discharge produced by wild-type mice preparations at E18.5 were regular and at a frequency similar to newborn pups. In marked contrast, the motor patterns generated by the preparations from *Ndn*^{tm2Stw} mice were very irregular with prominent bouts of depression of respiratory rhythmogenesis that would account for the hypoventilation observed in newborn *Ndn*^{tm2Stw} mice *in vivo*. The abnormal respiratory discharge pattern was present at the level of the diaphragm, cervical ventral roots, cranial motoneuron pools and within neurons located in the putative respiratory rhythm generating center, the pre-Bötzinger complex.

These data indicate that the defect in *Ndn*^{tm2Stw} mutant mice can be explained by abnormal respiratory rhythmogenesis emanating from the medulla. Data from *in vitro* (Smith et al., 1991) and *in vivo* (Gray et al., 2001; Ramirez et al., 1998; Solomon et al., 1999) models strongly suggest that a well-defined region of the ventrolateral medulla, the pre-Bötzinger complex, is a major contributor to the genesis of respiratory rhythm. A detailed understanding of the cellular mechanisms underlying rhythm and pattern generation with the ventrolateral medulla remains to be elucidated. However, there are data to support a pacemaker-network hypothesis, which states that the kernel for rhythm generation consists of a population of neurons with intrinsic pacemaker properties that are embedded within, and modulated by, a neuronal network (Rekling and Feldman, 1998; Smith et al., 2000). It has been postulated that the pacemaker properties arise from intrinsic voltage-dependent conductances that confer increases in burst frequency at depolarized membrane potentials and decreases, to the point of inhibiting rhythmic bursting, at hyperpolarized membrane potentials (Smith et al., 1991; Butera et al., 1999a & b). The primary conditioning excitatory drive that maintains the oscillatory state arises from activation of glutaminergic receptors (Greer et al., 1991; Funk et al., 1993). Further conditioning is provided by a diverse group of neuromodulators including GABA, serotonin, noradrenaline, opioids, prostaglandins, Substance P and acetylcholine (Lagercrantz, 1987; Moss & Inman, 1989; Ballanyi et al., 1999). Thus, absence of necdin expression could result in the loss, or perturbation of function, of rhythmogenic neurons in the pre-Bötzinger complex. This is the proposed abnormality in *Rnx*-deficient mice, which also have a central respiratory defect, possibly due to altered cell-fate commitment of respiratory neurons due to loss of this homeobox transcription factor (Shirasawa et al., 2000; Qian et al., 2001). Alternatively, necdin expression may be necessary for the proper functioning of neurons providing appropriate conditioning drive impinging on rhythmogenic neurons within the pre-Bötzinger complex. The later hypothesis was supported by the observation that the frequency of the respiratory rhythm could be increased and periods of apnea alleviated by the administration of SP, TRH, 5-HT and noradrenaline. However, the fluctuations in the respiratory frequency continued in the Ndn^{tm2Stw} mice. Our interpretation is that the exogenous application of neuromodulators overcame much of the deficit resulting from the abnormalities in medullary structures that normally provide conditioning drive to the preBötC. An abnormality in the function of the preBötC per se remains, however. The functional defect could reflect changes in neuronal properties or abnormalities in the preBötC network connectivity due to problems with axon guidance and fasciculation (Pagliardini et al., 2005).

People with PWS are deficient for multiple genes, including *necdin*. Although many aspects of PWS can be related to a basic defect in hypothalamic development, development of other systems is probably also compromised in PWS. Abnormal ventilatory responses to hyperoxia, hypoxia and hypercapnia when awake and sleeping are noted in PWS patients (Arens et al., 1994; Gozal et al., 1994; Schluter et al., 1997; Menendez, 1999). Further, there are reports of sleep-related central and obstructive apnea (Clift et al., 1994; Wharton & Loechner, 1996; Manni et al., 2001; Nixon & Brouillete, 2002). A report of a 29-week premature infant with PWS who required prolonged ventilatory support points to a prenatal onset of respiratory dysfunction in PWS (MacDonald & Camp, 2001). The sleep related breathing problems likely contribute significantly to the excessive daytime sleepiness in childhood and adulthood that is characteristic of PWS (Hertz et al., 1995). Aside from one report showing reduced number of oxytocin neurons in the hypothalamic paraventricular nucleus, no abnormal pathological findings have been noted in PWS individuals at autopsy (Swaab et al., 1995). Our study now suggests that loss of *necdin* is implicated in abnormal respiration in PWS infants, and we hypothesize that *necdin* may be important for normal respiratory activity in the human newborn medulla.

Table 1. Characterization of inspiratory bursts in wild-type and mutant embryonic mouse preparations

	n	Interval(s)	Duration(s)	Amplitude(mV)	Coefficient of variation of burst interval
Wild-type en bloc	5	3.2±2.3	0.32±0.09	46±21	0.72
Mutant en bloc					
Low frequency	7	32±41*	0.33±0.07	51±34	1.3*
Medium frequency	7	3.3±2.4	0.28±0.05	37±26	0.73
Combined average	7	8.5±17.3*	0.28±0.06	38±27	2.0*
Wild-type slice	5	3.5±2.8	0.30±0.08	33±18	0.8
Mutant slice					
Low frequency	8	24±37*	0.29±0.08	37±21	1.5*
Medium frequency	8	3.8±2.8	0.26±0.06	27±20	0.74
Combined average	8	7.1±15.6*	0.27±0.08	29±20	2.2*

The mean interburst interval, duration and amplitude of inspiratory bursts were calculated from recordings of inspiratory motor discharge generated by brainstem-spinal cord (en bloc) and medullary slice preparations from E18.5 mice. The measurements for mutant mice were calculated separately for bouts of low and medium frequency bursting, and put together as a combined average. Results are means ± SD; n is the number of preparations examined. *$p<0.05$ compared with wild-type; Student's t-test.

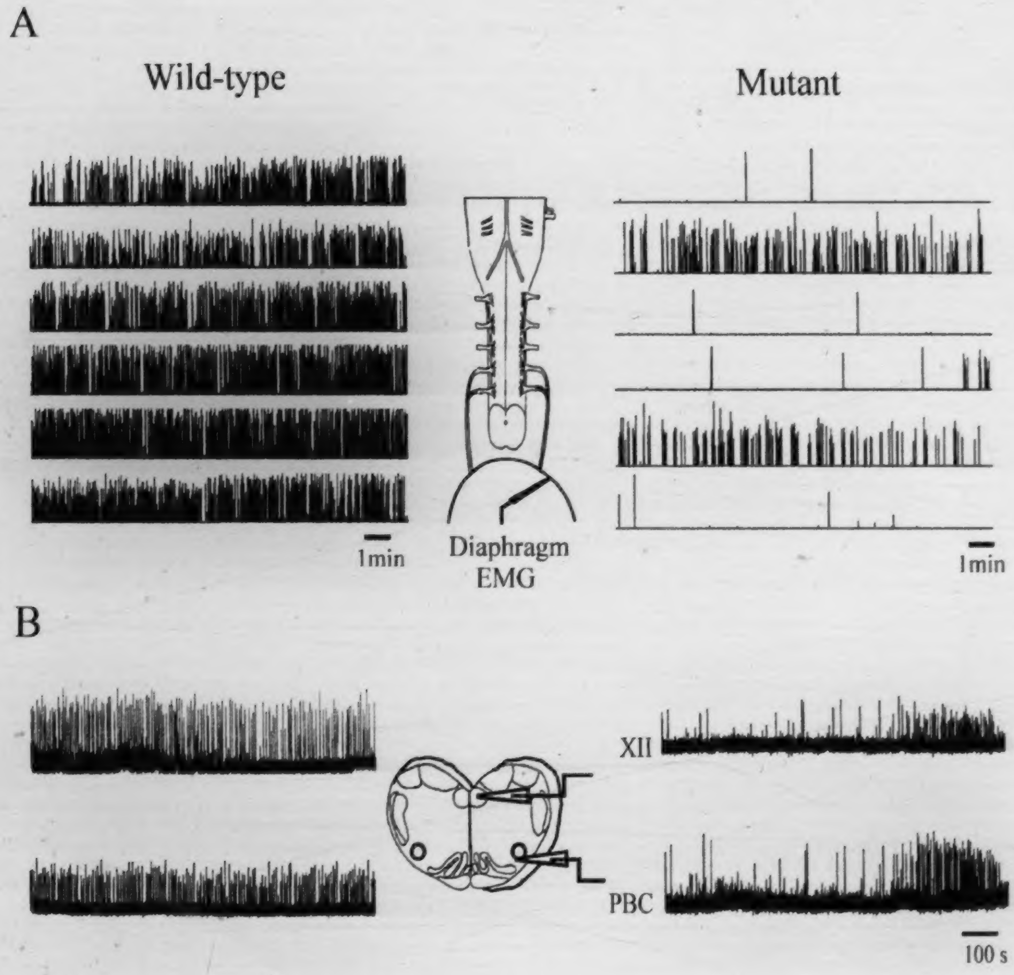
A
Wild-type
Mutant
1min
Diaphragm
EMG
1min
B
XII
PBC
100 s

Figure 4.1 ***Necdin*****-deficient (*****Ndn^{tm2Stw}*****) mice have irregular respiratory rhythms with prolonged periods of central apnea.** A) Sample rectified and integrated suction electrode recordings of diaphragm EMG were made from brainstem-spinal cord-diaphragm preparations isolated from E18.5 wild type (left panel) and *Ndn^{tm2Stw}* mutant (right panel) mice. Recordings of 80-minute duration demonstrate the regularity of respiratory discharge frequency (~4-5 sec interspike interval) in wild-type preparations. In contrast, the respiratory frequency is very unstable in mutant preparations over time. B) Defects in respiratory rhythm are observed within the putative respiratory rhythm-generating center. Sample rectified and integrated suction electrode recordings were made from inspiratory neurons located in the pre-Bötzinger complex (PBC) and neurons within the hypoglossal (XII) nucleus in medullary slice preparations isolated from E18.5 wild-type (left panel) and *Ndn^{tm2Stw}* mutant (right panel) mice.

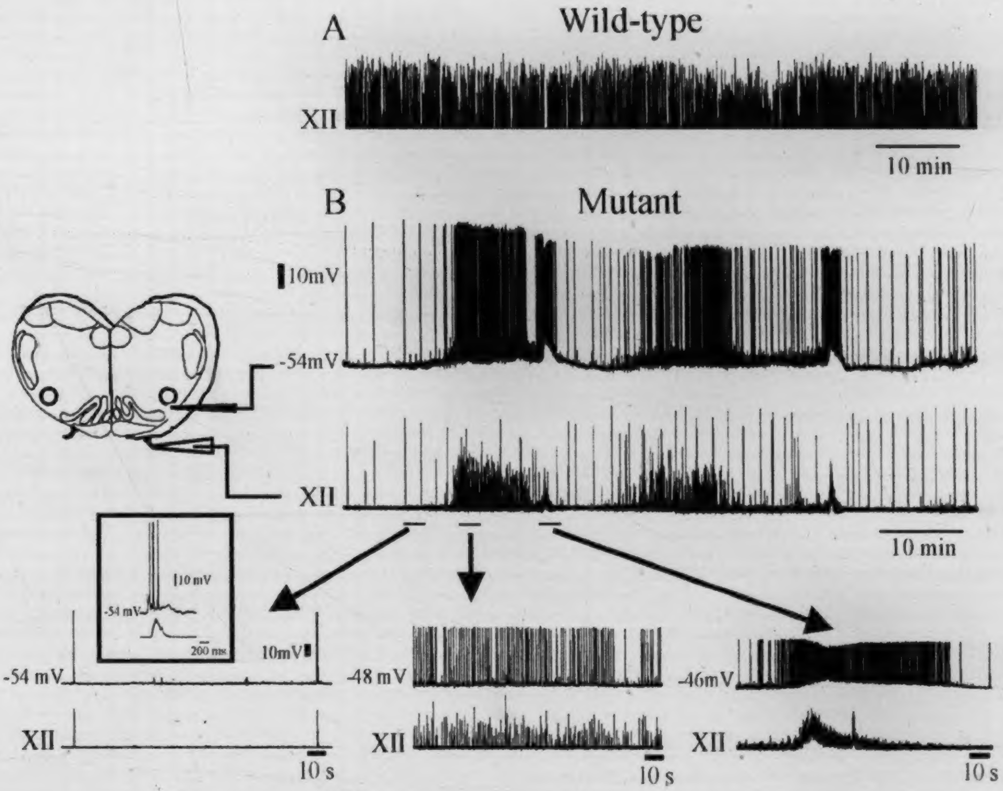

Figure 4.2 Abnormal rhythmogenesis is apparent from whole-cell patch-clamp recordings from an inspiratory neuron within the pre-Bötzinger complex. A) Rectified and integrated suction electrode recordings were made from the XII nerve roots of a wild-type E18.5 medullary slice preparation. B) Top panel shows whole-cell patch clamp recording from an inspiratory neuron located within the region of the pre-Bötzinger complex from an E18.5 *Ndn*^{tm2Stw} mouse. Middle panel shows the simultaneous recording from the XII nerve root. The bottom panel shows the whole-cell and nerve root recordings on a shorter time scale. The traces were taken from the areas demarcated on the middle panel with horizontal bars. The rhythmic discharge fluctuates between periods of very slow rhythms (left bottom panel) to those where the respiratory rhythm is similar in frequency to wild-type preparations (middle bottom panel). There are also occurrences of high frequency non-respiratory bursts (right bottom panel). Insert shows whole-cell and integrated nerve recordings during a single inspiratory burst.

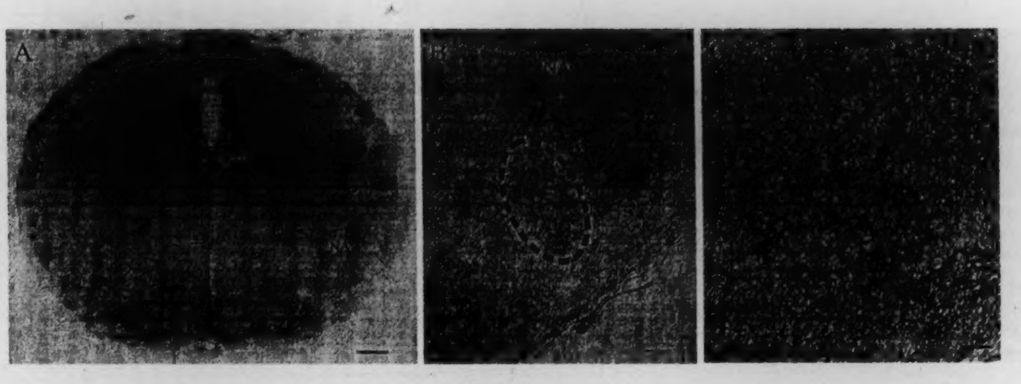

Figure 4.3 Necdin is expressed in the fetal medulla. A) Expression of *Ndn* in E18.5 medullary transverse section equivalent to those used for electrophysiological studies. B) Photo of labeling in the ventrolateral medulla (preBötzinger complex area approximated by dashed line). C) Higher power photo of the preBötzinger complex region. Structures are as follows: NA, nucleus ambiguous; X, dorsal motor nucleus of the vagus nerve; XII, nucleus of the twelfth nerve (hypoglossal). Scale bars: A = 200 μm; B = 50 μm; C = 50 μm

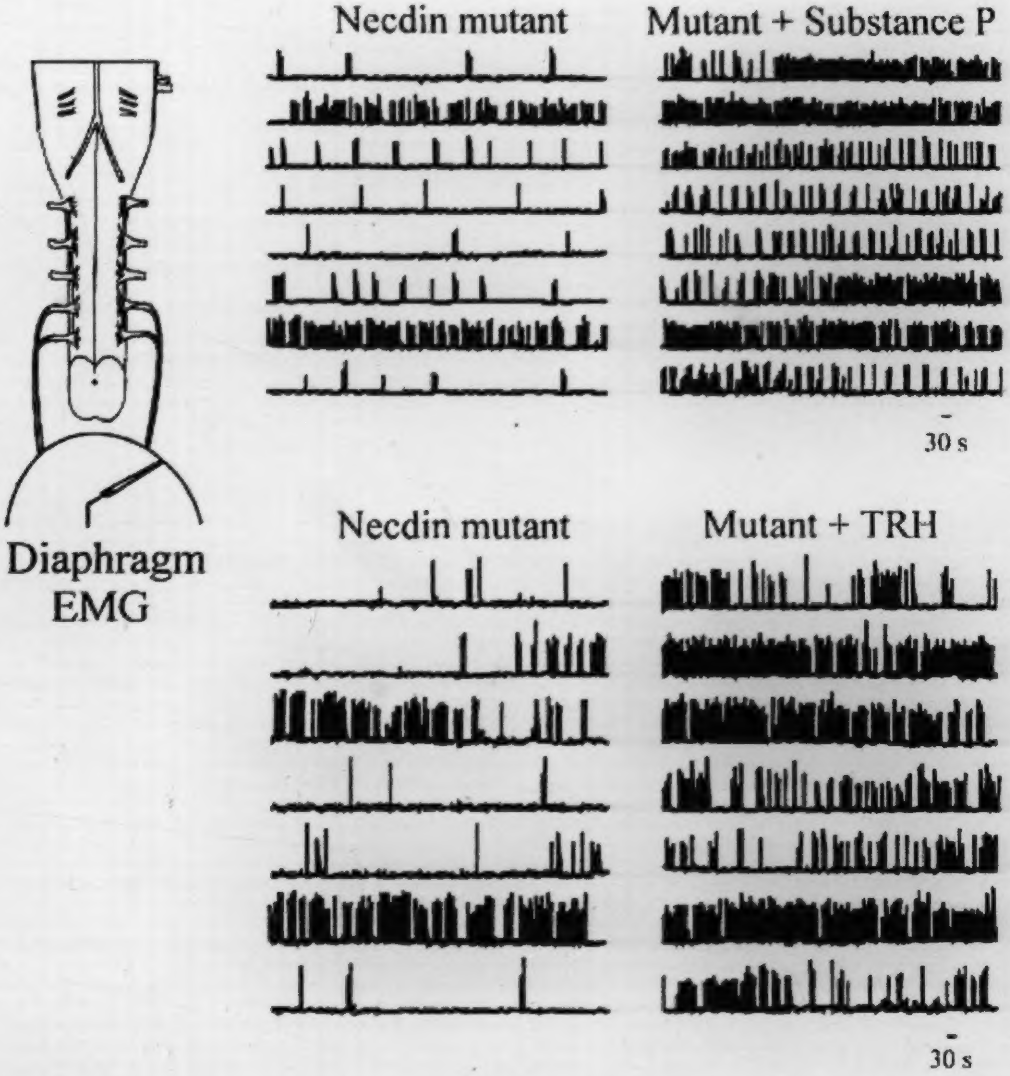

Figure 4.4 Effects of excitatory neuromodulators on respiratory rhythm generated by Ndn^{tm2Stw} mouse brainstem-spinal cord preparations. Left panel illustrates the isolated brainstem-spinal cord-diaphragm *in vitro* preparation. Right panel shows rectified and integrated suction electrode recordings of diaphragm EMG from E18.5 Ndn^{tm2Stw} mice in control solution (left) and in response to the addition of SP (1 μm) and TRH (1 μm) to the bathing medium (right). Both neuromodulators increased the overall frequency of respiratory rhythm but the instabilities in the frequency remained.

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CHAPTER V

AMPAKINE CX546 ALLEVIATES RESPIRATORY DEPRESSION IN RATS

*Previously published paper:

Ren J, Poon BY, Tang Y, Funk GD, Greer JJ (2006) Ampakines alleviate respiratory depression in rats. Am J Respir Crit Care Med 174(12):1384-91. Copyright 2006 by American Thoracic Society.

My contribution to this study consisted in the planning and execution of the *in vitro* and *in vivo* study. Recordings and analyses from *in situ* working heart preparations were performed by Poon BY and Funk GD.

5.1 INTRODUCTION

There is a concerted effort toward understanding the neurochemical control of respiratory rhythm generating networks as well as the premotor and motoneuron circuits that determine activity patterns of respiratory muscles. Insights derived from experimental work will be important for developing pharmacological interventions to alleviate respiratory depression and apneas. A specific region within the ventrolateral medulla, the preBötzinger complex (preBötC), plays a critical role in generating rhythmic inspiratory drive, and thus is a major region of investigative focus (Feldman & Del Negro, 2006). Within the preBötC, glutamatergic synaptic signaling mediated by non-NMDA receptors (primarily AMPA receptors) is particularly important for maintaining respiratory rhythm (Greer et al., 1991; Funk et al., 1993). Block of non-NMDA receptors with the antagonist CNQX causes a dose-dependent decline, and eventual cessation, of respiratory frequency and inspiratory drive to cranial and spinal motoneurons. Elevation of endogenously released glutamate levels with glutamatergic uptake inhibitors (Greer et al., 1991) or reduction of AMPA receptor desensitization (Funk et al., 1995) leads to increases in respiratory frequency *in vitro*. In this study, we test the hypothesis that the ampakine CX546 provides a pharmacological means to counteract respiratory depression. This drug is a member of the ampakine family of compounds that modulate the AMPA receptor complex by increasing the duration of glutamate-induced AMPA receptor-gated inward currents (Arai et al., 2004). Specifically, CX546 binds to the receptor in its agonist-bound, but not the desensitized or agonist-unbound states, and modulates the kinetics of deactivation (channel closing and transmitter dissociation) and desensitization (Nagarajan et al., 2001). The combined use of *in vitro*, *in situ* and *in vivo* rat models allowed for the analyses of CX546 on preBötC activity and respiratory motor pools in reduced and intact preparations across multiple developmental stages.

5.2 MATERIAL AND METHODS

5.2.1 *IN VITRO* PREPARATIONS AND *IN VITRO*/ *IN VIVO* RECORDINGS

See Chapter 2.

5.2.2 PERFUSED HEART *IN SITU* PREPARATION

Methods are described in detail elsewhere (Paton, 1996; Day & Wilson, 2003). In brief, juvenile SD rats (between 3 and 4 weeks of age, 80-120 g) were anesthetized with isoflurane, submerged in ice-cold oxygenated perfusate, decerebrated, and transected caudal to the diaphragm and the descending aorta cannulated (<8 min). The torso and brainstem were then transferred to a recording chamber where the descending aorta was cannulated with a double-lumen cannula (one line to deliver perfusate, the second to monitor blood pressure) and perfused with saline (bubbled with 95% O_2/5% CO_2) at a flow rate sufficient to generate and maintain arterial pressure at 60 mmHg. Once perfusion was initiated and arterial pressure stabilized, the animal was gradually warmed to 32°C by heating the perfusate. The preparation was then allowed to stabilize for one hour (from the start of the dissection), during which time the left phrenic nerve was dissected for monitoring inspiratory activity (frequency and burst amplitude). After the one hour stabilization period, baseline respiratory output was recorded and the various drugs (fentanyl and CX546) added directly to the perfusate.

5.2.3 NOCICEPTIVE TESTING

Thermal nociception was measured by a modification of a previously reported method (13). Briefly, unrestrained rats were placed in a plastic chamber (14 x 16 x 22 cm) and allowed to acclimate for 10 min before testing. The plantar test apparatus (Ugo Basile, Comerio, VA, Italy) consisted of a movable infrared heat source (OSRAM 8V 50W halogen lamp) which was positioned directly beneath the hind paw, 20 mm below the chamber floor. Heat settings were 30 and 50 (manufacturer settings) for newborn and adult rats, respectively. The instrument detected paw withdrawal latency from the onset of heat exposure, with an accuracy to 0.1 sec. When the rat perceived pain and withdrew its paw, the instrument automatically detected the withdrawal latency to the nearest 0.1 s. Withdrawal latencies were recorded before, during, and after each drug administration. The heat stimulus was automatically terminated if a withdrawal response was not observed within 20 sec of its onset.

5.2.4 PHARMACOLOGICAL AGENTS

All drugs were purchased from Sigma (St. Louis, MO). The ampakine CX546 was dissolved in dimethylsulfoxide (DMSO) to make a 50-200 mM stock solution. CX546 (50-400 μM) and the μ-opioid receptor agonist DAGO (800 nM) were used *in vitro* and added directly to the bathing medium. Fentanyl (4 nM) and CX546 (50 μM were added to the perfusate of the working heart *in situ* preparation. For the *in vivo* plethysmographic studies, CX546 was administered intraperitoneally (i.p.) at a dosage of 16 mg/kg (dose based on Lauterborn et al., 2000). Fentanyl HCl (60 μg/kg for newborn and 130 μg/kg for adult rats) and phenobarbital (28 mg/kg for newborn and 100 mg/kg for adult rats) were dissolved in physiological saline and administered i.p. (5-10 μl total volume for newborn and 150-300 μl for adult rats) to reduce baseline respiratory frequency by ~50%. Administration of vehicle did not affect the respiratory parameters studied in any preparation.

5.2.5 ANALYSIS

For *in vitro* and *in situ* experiments, values of respiratory period (or frequency) and peak inspiratory burst amplitude were measured from the integrated nerve recording and reported as means relative to control values. For *in vivo* plethysmography, inter-breath intervals and the relative amplitude of volume excursions associated with each breath were calculated before and after (5 minutes) i.p. drug administration. In all cases, values are given as means and standard deviations. Statistical significance was tested using Student's t test for paired or unpaired data (two groups) or one way repeated measures ANOVA (multiple groups) followed by Holm-sidak test for multiple comparisons. Significance was accepted at p values lower than 0.05.

5.3 RESULTS

5.3.1 *IN VITRO* PERINATAL PREPARATIONS

The fetal and neonatal brainstem-spinal cord preparations have been well-characterized and shown to generate complex, coordinated patterns of respiratory-related activity (Smith et al., 1990; Greer et al., 1992). Recordings from cervical ventral and hypoglossal cranial roots provide information regarding the pharmacology of respiratory rhythm generating networks and the pathways transmitting that respiratory drive to key output components of the respiratory motor system, without the confounding influence of peripheral chemoreceptors and supramedullary structures. Fig 5.1A shows a representative example of the respiratory discharge produced by an embryonic day (E)20 brainstem-spinal cord preparation. The frequency of rhythmic respiratory discharge was markedly enhanced by the addition of CX546 (50-100 μM) to the bathing medium. Population data showing the increase in respiratory frequency compared to control for ages E18-P3 are provided in Fig 5.1B. In perinatal preparations (E18-P0), that often have a slower baseline rhythm compared to older neonates (Greer et al., 1992), CX546 caused a significant increase in frequency. By P3, however, CX546 had no effect on the baseline frequency of the more robust respiratory output generated by these older brainstem-spinal cord preparations.

The medullary slice preparation is a derivative of the brainstem-spinal cord preparation (Smith et al., 1991). It contains the minimum component of neuronal populations within the ventrolateral medulla necessary for generating a respiratory rhythm, the preBötC. The medullary slice also contains a significant portion of the rostroventral respiratory group, hypoglossal nucleus and XII cranial nerve rootlets from which inspiratory motor discharge is recorded. Fig 5.2 illustrates the effects of CX546 on the respiratory rhythm generated by medullary slice preparations. The respiratory-related output generated by these slices typically shows a gradual decrease in frequency and burst amplitude and stops within approximately 60 min when medullary slices are prepared and bathed in solution containing 3 mM $[K^+]_o$. Bath application of CX546 (400 μM) to the slices after rhythmic activity ceased in 3 mM $[K^+]_o$ caused a rapid and potent stimulation of respiratory networks. Within 2 minutes of CX546 application, frequency and amplitude were restored to levels as, and in some cases, greater than observed at any point in bathing media containing 3 mM $[K^+]_o$.

The next series of experiments examined the ability of CX546 to counter the depression of respiratory frequency and amplitude caused by the μ-opioid receptor agonist DAGO. Figs 5.3A&B show recordings of rhythmic respiratory discharge generated by P1 brainstem-spinal cord and medullary slice (bathed in 9 mM $[K^+]_o$) preparations. DAGO (800 nM) markedly suppressed respiratory frequency and amplitude in both preparations. The DAGO-induced depression was alleviated by the subsequent administration of CX546 (200 μM). Population data are provided in Fig 5.3C&D. The administration of CX546 on its own did not significantly alter the control values of respiratory frequency or amplitude of motor nerve discharge generated by medullary slice preparations bathed in 9 mM $[K^+]_o$.

5.3.2 PERFUSED HEART *IN SITU* DATA

Rhythmically-active, brainstem-spinal cord, and medullary slice preparations in rat are not viable beyond the newborn period. However, one can examine central respiratory control and its pharmacology in reduced preparations of older rodents using the *in situ* working heart-brainstem preparation (Paton, 1996). These preparations generate a normal (*in vivo*-like) breathing motor pattern which includes augmenting phrenic bursts. They are also oxygenated throughout and have uniform brain tissue pH (Wilson et al., 2001). Fig 5.4A shows a representative example of phrenic nerve discharge recorded from a P24 rat *in situ* preparation. Administration of fentanyl (4 nM) to the perfusate produced a significant decrease of respiratory frequency and phrenic burst amplitude that was countered by subsequent administration of CX546 (50 μM). Population data are presented in Fig 5.4B.

5.3.3 *IN VIVO* PLETHYSMOGRAPHY

The final stage of the study was to examine the actions of CX546 *in vivo*. Whole-body plethysmography was used to examine the breathing patterns generated by unanesthetized newborn (P0-P2) and adult rats. Respiration was depressed by either i.p. administration of the μ-opioid receptor agonist fentanyl or the barbiturate phenobarbital. As shown in representative examples in Fig 5.5A,B&C and in the population data of Fig 5.5D,E&F, both agents significantly decreased respiratory frequency and burst amplitude. Subsequent i.p. injection of CX546 (16 mg/kg) reversed the opiate- and phenobarbital-mediated respiratory depression. The baseline frequency and amplitude of newborn (P0-P2, n=5) and adult rats (n=3, data not shown), however, were not significantly altered by the same dose of CX546 on its own. CX546 was also without any obvious effect on the behaviour or of arousal-state (i.e. increase/decrease in spontaneous movement, agitation, indication of sedation) of the rats.

5.3.4 NOCICEPTIVE TESTING

The above experiments demonstrated that CX546 counteracts the respiratory depression induced by DAGO or fentanyl. We then investigated whether the i.p. administration of CX546 affects fentanyl-induced analgesia *in vivo*. Using a thermal nociception test, the latency of hind paw withdrawal was 4.9±3.8s and 5.6±1.7s for newborns and adult rats, respectively (see Table 5.1). Administration of the fentanyl at doses that suppressed respiratory rhythm (60 μg/kg for newborn and 130 μg/kg for adult rats) extended the latency to paw-withdrawal to the 20 sec cut-off limit. This fentanyl-induced analgesia, which manifest in the loss of foot withdrawal, persisted despite the subsequent i.p. administration of CX546 (16 mg/kg). The fentanyl-induced analgesic effect, was however, blocked by the subsequent administration of naloxone (1 mg/kg) in both newborn and adult rats. When CX546 (16 mg/kg) was administrated on its own, it did not change the sensitivity of thermal nociception tests in the control condition. These results demonstrate that CX546 reduces the deleterious respiratory-depressant effects of opioids without inhibiting their desirable, analgesic actions.

5.4 DISCUSSION

Respiratory depression has serious clinical implications and manifests in a number of situations including pharmacologically-induced and endogenous bradypnea and apneas (central and obstructive). Advancements in drug treatments based on an understanding of the neurochemical control of breathing are needed. Here, we demonstrate that an ampakine, which potentiate AMPA receptor-mediated conductances (Nagarajan et al., 2001) critical for inspiratory rhythmogenesis and synaptic drive to respiratory motor nuclei, is an effective means of countering respiratory depression.

5.4.1 AMPAKINE-MEDIATED MODULATION OF RESPIRATORY ACTIVITY

5.4.1.1 SITES OF ACTION

The administration of the ampakine CX546 provided a powerful and robust means of alleviating depressed central respiratory drive; respiratory activity was enhanced when the network was examined in its most reduced (medullary slice) and most intact (*in vivo*) states. Potentiation of output in medullary slices and the brainstem-spinal cord preparations indicates that CX546 acts on medullary respiratory networks. Direct action within the preBötC rhythm generating networks is likely since frequency is enhanced. However, it is also possible that the increased frequency reflects secondary actions through a population of neurons that provide tonic excitatory glutamatergic drive to the rhythm generator. Amplitude effects *in vitro* and *in vivo* also suggest that premotor and motoneuron pools are sites of ampakine action. Certainly, inhibition of desensitization, which is one effect of ampakines, within the XII motor nucleus potentiates inspiratory burst amplitude (Funk et al., 1995).

The observation that the potentiating actions of ampakines are also present *in situ* and *in vivo*, and therefore not limited to the *in vitro* preparations, indicates that potentiation is not dependent on some unique condition established *in vitro*. Ampakines counteract respiratory depression in the presence of excitatory drive to the central respiratory rhythm generation from peripheral chemoreceptors, mechanoreceptors, and supramedullary centers. In fact, it is possible that in more intact preparations, ampakines act not only within the preBötC and motor pools, but also enhance activity in excitatory afferent feedback pathways.

5.4.1.2 DEVELOPMENT

Ampakine-mediated enhancement of respiratory activity was observed in animals ranging in age from E18 to adult. In animals P0 or younger, ampakines enhanced baseline activity. In older preparations, ampakines only enhanced activity if respiration was initially depressed. Whether or not this reflects a developmental change in sensitivity to ampakines or simply that the potentiating actions of ampakines only manifest if respiratory activity is first suppressed is unclear. Paradoxically, if ampakines potentiate respiratory output by blocking desensitization, one would predict greater efficacy in older animals with conditions of increased network activity due to increased glutamate receptor activation and the greater potential for desensitization. Further, the inhibition of desensitization kinetics by CX546 should become more significant with maturation. AMPA receptor subunits, GluR1-4, exist as either flip or flop splice variants. In embryonic animals, the flip variants predominate (Seeburg, 1993). With maturation there is a shift in expression such that the flop isoforms, which have more rapid desensitization kinetics, become increasingly dominant. Thus, these data suggest that the most significant action of ampakines in relation to controlling activity of respiratory networks may not be modulation of glutamate receptor desensitization properties. Rather, enhancement of binding affinity and prolongation of channel open time may be the important parameters.

Defining the molecular mechanism(s) underlying the ampakine-mediated enhancement of depressed respiratory output will require analysis at the single-cell and most likely single-channel levels, and is beyond the scope of this study. Despite an incomplete mechanistic understanding, potentiation of depressed but not baseline activity is a very desirable feature of ampakine action from a clinical perspective. Enhancing baseline ventilation or increasing depressed respiratory output to levels greater than baseline (i.e. hyperventilation) would have independent deleterious consequences including the disruption of blood gases, and predisposition to respiratory instability.

5.4.1.3 SPECIFICITY OF ACTION ON RESPIRATORY NETWORKS

The finding that CX546 stimulated breathing without wide-spread activation of neuronal circuits may not be expected given the ubiquitous distribution of AMPA receptors within the central nervous system. However, behavioral selectivity of ampakines has been reported in other studies and might be explained by regional and network selectivity of ampakines (Lynch, 2006). AMPA subunits (GluR1-4 subunits, each with flip and flop splice variants and RNA-editing R/G and Q/R site; Seeburg, 1998) are differentially expressed throughout the brain. Ampakines have subunit-specific effects and could act with regional selectivity. For example, ampakines have a several-fold greater effect on excitatory post-synaptic currents in hippocampus (high concentrations of GluR1-2) than in thalamus (GluR3-4). Even within the hippocampus, ampakines have very different effects across subgroups of cells. It's therefore possible that ampakines have particularly strong effects on the neurons regulating respiration.

5.4.1.4 ADDITIONAL AMPAKINE ACTIONS

In addition to their direct effects on glutamatergic signaling, ampakines, including CX546, appear to chronically elevate the production of BDNF in hippocampal and cortical neurons via the positive modulation of AMPA receptors (Lauterborn et al., 2000). However, the rapid kinetics of the ampakine CX546 treatment on respiratory drive is inconsistent with an up-regulation of BDNF synthesis. Moreover, BDNF decreases, rather than increases, the frequency of respiratory rhythm in medullary slice preparations (Thoby-Brisson et al., 2003). A further mechanism of CX546 action is the enhancement of astrocyte metabolism which increases glucose utilization and lactate production (Pellerin & Magistretti, 2005). The possibility of such astrocyte-mediated actions influencing respiratory drive, in conjunction with direct modulation of neuronal AMPA receptor conductances, should be considered given the proposed metabolic coupling between glia and medullary respiratory neurons (Hulsmann et al., 2000). However, the acute actions of CX546 on respiratory frequency are unlikely to solely reflect glia-derived increases in extracellular lactic acid because bath application of lactic acid (5 mM) to the brainstem-spinal cord or medullary slice preparations did not mimic the actions of CX546 (Ren and Greer, unpublished observation).

5.4.2 IMPLICATIONS FOR ANALYSIS OF RESPIRATORY RHYTHM GENERATION

The first series of experiments was performed using *in vitro* brainstem-spinal cord and medullary slice preparations. These *in vitro* preparations isolated from embryonic or P0 rats typically generate a respiratory rhythm of low frequency compared to older neonatal animals (reviewed in Greer et al., 2006). This is in part due to lack of sufficient excitatory drive from glutamatergic and neuromodulatory systems. Data from this study demonstrate that CX546, presumably via the modulation/potentiation of the AMPA receptor conductance, markedly enhances the frequency of respiratory rhythm *in vitro* in E18-P0 rats, and restores depressed rhythm in older neonates in both the brainstem-spinal cord and medullary slice preparations.

Of significance is that the medullary slice preparation produces robust respiratory-related rhythm when perfused with bathing medium containing 3 mM $[K^+]_o$ and CX546. The rhythmic slice preparation has become a standard model in respiratory control studies and its use has significantly advanced understanding of the sites and mechanisms of mammalian rhythm generation (Smith et al., 1991). A limitation of these slice preparations, however, has been the necessity of bathing the tissue in elevated $[K^+]_o$ (typically 9 mM) for generation of long-lasting rhythmic activity (although see Poon et al., 2005; Ruangkittisakul et al., 2006). The perceived need for elevated $[K^+]_o$ underlies the argument of some investigators that mechanisms of rhythm generation identified *in vitro* are not relevant to breathing *in vivo*. The opposing view, presented with the original description of the slice (Smith et al., 1991; Funk et al., 1993), is that the elevated $[K^+]_o$ is required in the slice to compensate for the loss of tonic and phasic excitatory glutamatergic synaptic drive to rhythm-generating networks that is present in more intact *in vitro* preparations, such as the brainstem-spinal cord preparation, and under *in vivo* conditions. Accumulating evidence emphasizes the potential importance of $[K^+]_o$ in determining the effects of neuromodulatory cascades on rhythm, but they also suggest that many of the basic mechanisms of rhythm generation described *in vitro* apply *in vivo*. For example, the importance of the preBötC in rhythm generation, and the pacemaker behavior of preBötC neurons etc. are not dependent on elevated $[K^+]_o$ (Del Negro et al., 2005; Feldman & Del Negro, 2006). The demonstration in this study that ampakines evoke a robust, long-lasting respiratory rhythm at physiological $[K^+]_o$ adds further support to the thesis. It also provides an alternative to the use of elevated $[K^+]_o$ or addition of neurotransmitter receptor agonists for producing long-lasting rhythmic respiratory activity in medullary slices.

5.4.3 FUNCTIONAL/CLINICAL SIGNIFICANCE

The *in vivo* data from newborn and adult rats demonstrate that the actions of ampakines observed *in vitro* and *in situ* occur in the intact animal. Ampakines act as a powerful stimulant of respiratory frequency and tidal volume in perinates and rats of all ages in which respiration was depressed by the μ-receptor agonist fentanyl or the barbiturate phenobarbital. Opiates are widely used analgesics and phenobarbital is the longest-acting of the barbiturates, which are primarily used for sedation or as an anticonvulsant. However both have serious adverse effects, perhaps most significant is respiratory depression. Richter's group has demonstrated that a 5-HT4(a) receptor agonist counters opioid-induced breathing depression without loss of analgesia in rats

(Manzke et al., 2003; Richter et al., 2003). The mechanisms underlying 5-HT4(a) receptor functions as an antidote to fentanyl have not been clearly delineated although they may interact via convergent cAMP intracellular signaling. This is supported by the finding that nociceptin-induced respiratory depression *in vitro* is reversed by activation of adenylyl cyclase or block of phosphodiesterase activity (Ruangkittisakul & Ballanyi, 2006). It is important to note that the administration of CX546 countered the drug-induced respiratory depression without significantly altering the baseline frequency or amplitude of breathing in control animals, the inhibition of opiate-induced analgesia, or noticeably affecting the animals' behavior. Besides the effectiveness of ampakines to counter drug-induced respiratory depression, it will be of interest to determine if ampakines are effective in treating central and obstructive apneas in the newborn and adults; areas in which advancements in treatment are certainly needed (Millar & Schmidt, 2004; Smith & Quinnell, 2004). Genetic mouse models with clear central hypoventilation during the postnatal period (Gaultier & Gallego, 2005) should provide experimental opportunities to explore this area. Future studies will be necessary to determine the relative efficacy of various ampakines and to perform a more thorough examination for potentially harmful side-effects. Further, this study was limited to an examination of the acute actions of ampakines over minutes. A drug-delivery system that provides ampakine-mediated actions over longer periods will likely be necessary for uses beyond countering drug-induced respiratory depression.

5.4.4 CONCLUSION

Data from the current study indicate that ampakines may provide a novel pharmacological means of countering respiratory depression. Importantly, ampakines readily cross the blood-brain barrier and appear to be well-tolerated (Goff et al., 2001; Porrino et al., 2005; Lynch, 2006). Further work evaluating the efficacy of the various members of currently available and developing members of the ampakine family as respiratory stimulants toward applicability in clinical settings is warranted.

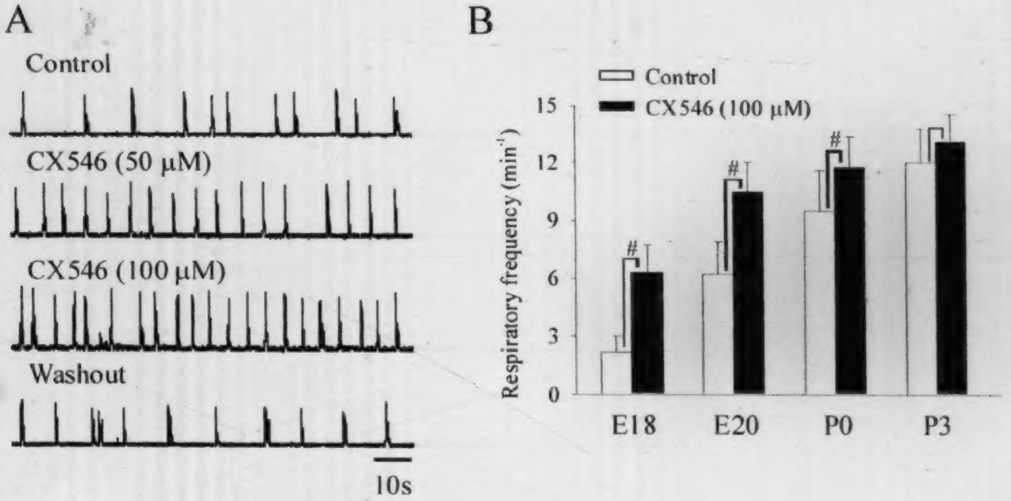

Figure 5.1 CX546 stimulates frequency of rhythmic respiratory activity generated by brainstem-spinal cord preparations. A) Rectified and integrated suction electrode recordings of C4 ventral root discharge from an E20 brainstem-spinal cord preparation in response to bath application of CX546. B) Population data showing changes in respiratory frequency of brainstem-spinal cord preparations in response to bath application of CX546 at different perinatal ages (n=4-5 for each age; # indicates significant difference between control and CX546; $p < 0.05$).

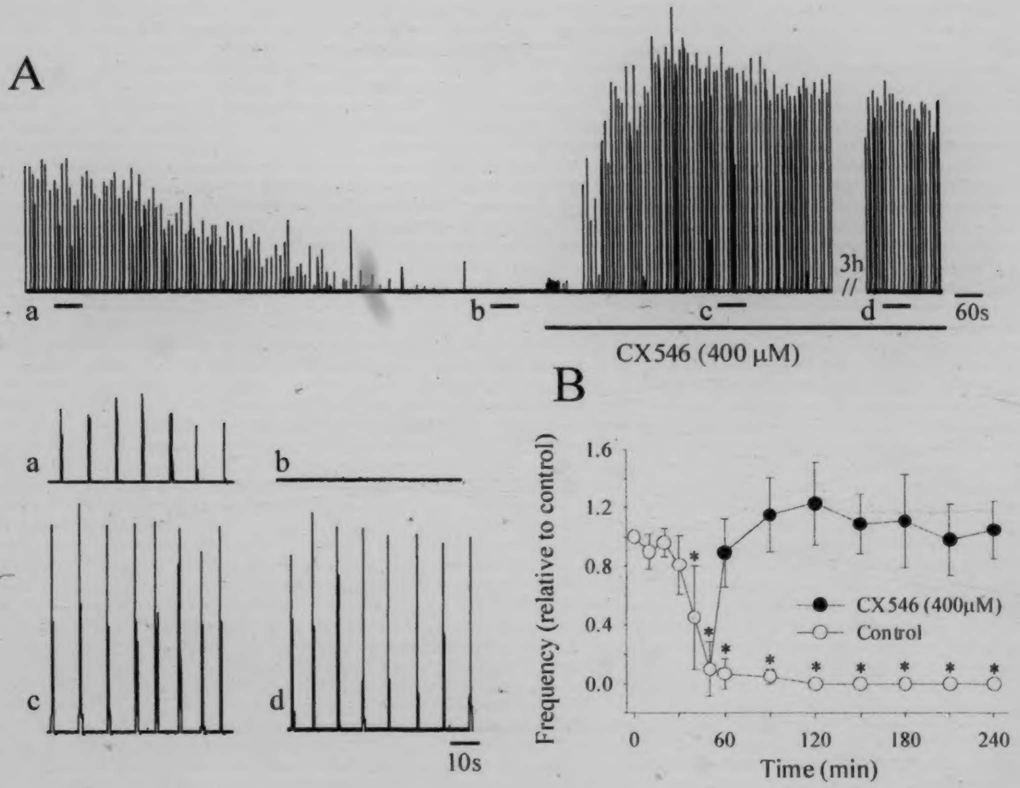
A
3h
//
a
b
c
d
60s
CX546 (400 μM)
a
b
c
d
10s
B
Frequency (relative to control)
1.6
1.2
0.8
0.4
0.0
0
60
120
180
240
Time (min)
CX546 (400μM)
Control
*

Figure 5.2 CX546 stimulates frequency of rhythmic respiratory activity generated by medullary slice preparations. A) Long-term recording (>3 hours) from a P2 medullary slice preparation perfused with bathing medium containing 3 mM $[K^+]_o$, showing rectified and integrated hypoglossal nerve root (XII) activity. The bottom traces in A (a-d) show expanded records of respiratory discharge at specific periods during the recording. Note that when perfused with 3 mM $[K^+]_o$ bathing medium, rhythmic activity was present shortly after slice production (a), but burst amplitude and frequency gradually diminished and stopped in <60 min (b). c - After cessation of rhythmic activity (in 3 mM $[K^+]_o$), bath-application of CX546 restored rhythmic activity of XII motoneurons. Moreover, the respiratory frequency and amplitude in CX546 were greater than under initial control conditions. d - Respiratory rhythm persisted for more than 3h in the presence of CX546 (longest period tested). B) Population data showing time course of changes in respiratory frequency (relative to control levels averaged over the first 2 min of recording) of P2 medullary slice preparations in 3 mM $[K^+]_o$ medium or 3 mM $[K^+]_o$ followed by addition of CX546 at t=60 min (n=3 for each data point post-45 minutes, n=6 0-45 minutes). * indicates significant difference relative to control.

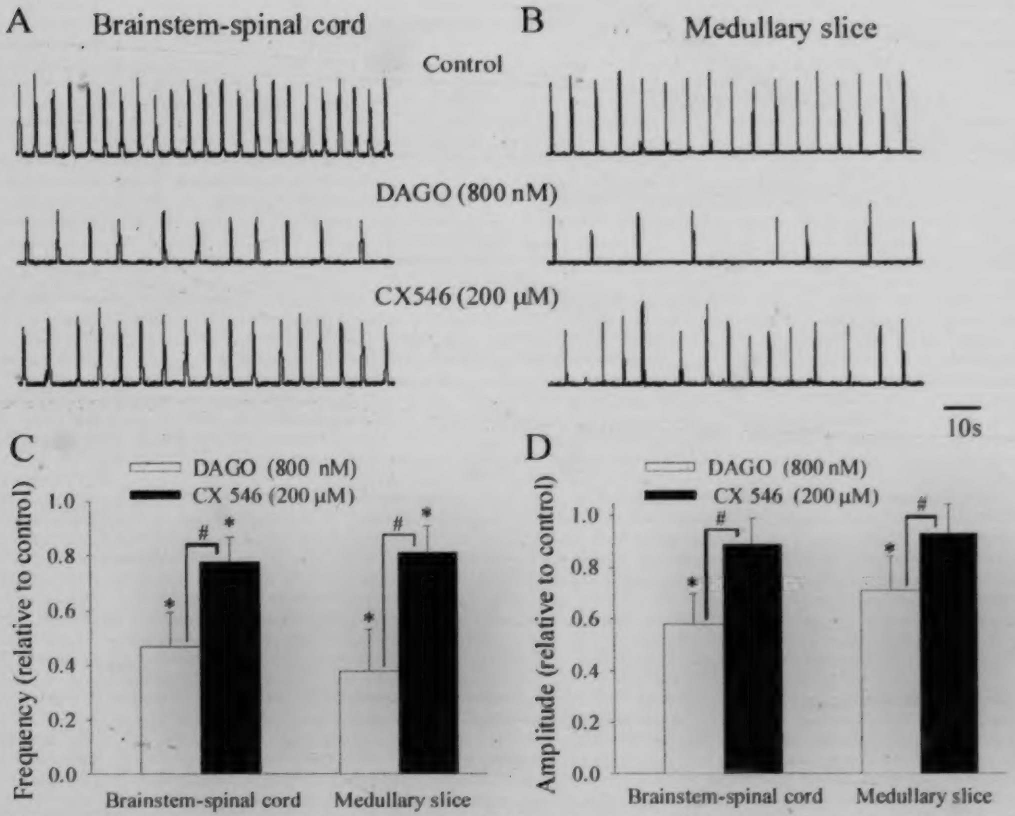

Figure 5.3 CX546 counters opioid-induced respiratory depression *in vitro*. Rectified and integrated recordings of A) C4 ventral roots (brainstem-spinal cord of a P1 rat) and B) XII nerve roots (medullary slice of a P1 rat perfused with 9 mM $[K^+]_o$ bathing medium) in response to bath application of the μ-opioid receptor agonist DAGO. The DAGO-induced suppression of respiratory frequency and amplitude in these preparations was partially reversed by the subsequent bath-application of CX546. C, D) Population data for brainstem-spinal cord and medullary slice preparations (P1-P2 rats; n=7 for each preparation) showing the effects of bath-applied DAGO alone and then CX546 (in the continued presence of DAGO) on inspiratory frequency and burst amplitude relative to control values (of 1.0). * indicates significant difference relative to control; # indicates significant difference between value in DAGO alone and after subsequent application of CX546. (Values of frequency and amplitude are reported relative to control values standardized to 1.0)

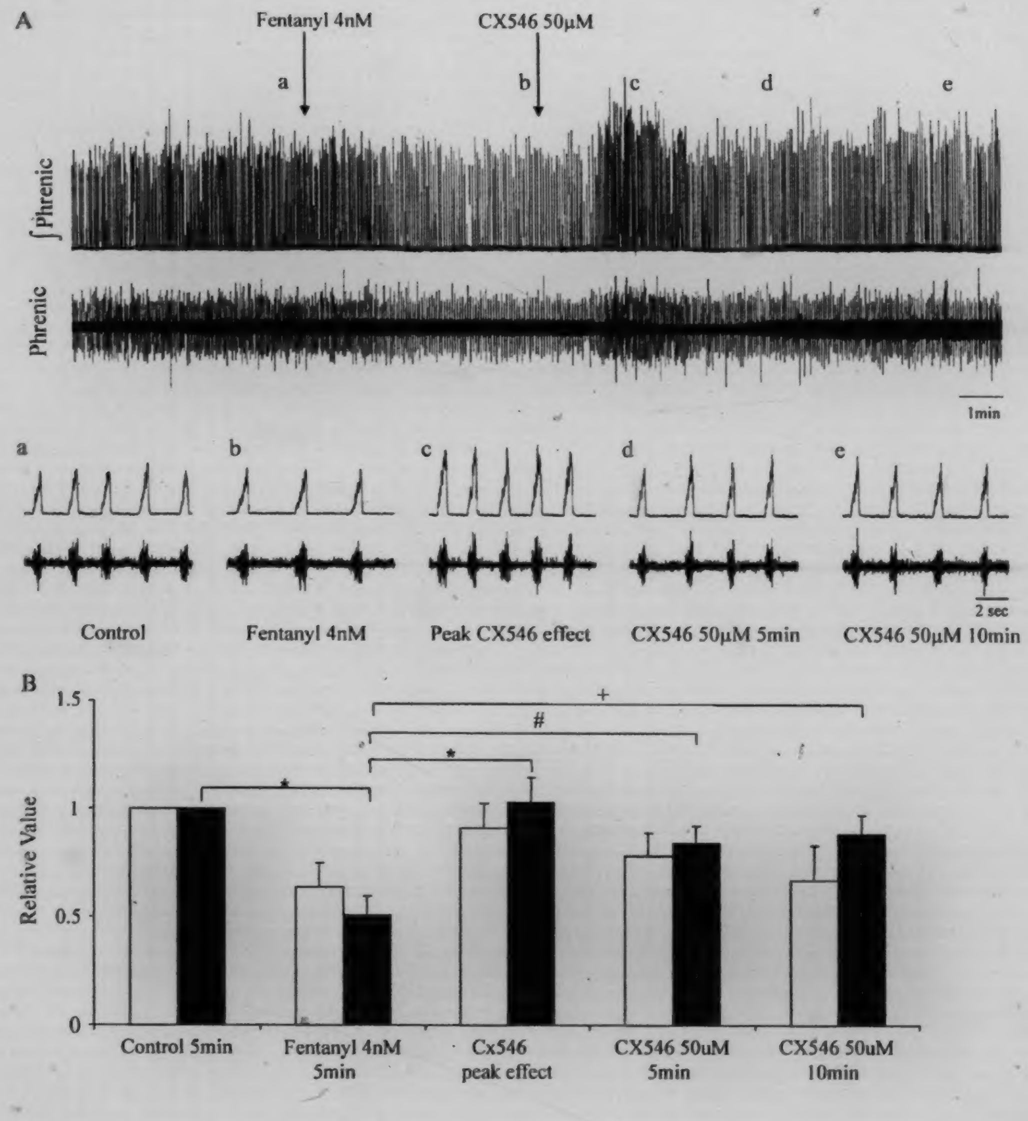
A
Fentanyl 4nM
CX546 50μM
a
b
c
d
e
∫Phrenic
Phrenic
1min
a
b
c
d
e
2 sec
Control
Fentanyl 4nM
Peak CX546 effect
CX546 50μM 5min
CX546 50μM 10min
B
+
#
*
*
1.5
1
0.5
0
Relative Value
Control 5min
Fentanyl 4nM 5min
Cx546 peak effect
CX546 50uM 5min
CX546 50uM 10min

Figure 5.4 CX546 counters opioid-induced depression of respiratory frequency and amplitude generated by perfused heart *in situ* preparations. A) Long-term recording of raw and integrated phrenic nerve discharge in a P24 rat. Time points at which fentanyl and CX546 were added to the reservoir containing oxygenated perfusion medium are indicated by arrows. Note that it took approximately 90 sec for solution in the reservoir to reach the preparation. Bottom panels show expanded sections of phrenic nerve respiratory discharge taken from the regions labelled a-e in the long-term recording. B) Population data (n=8) showing the relative effects (compared to control period) of fentanyl and then CX546 (in the continued presence of fentanyl) on frequency (open bars) and amplitude (filled bars) of integrated phrenic nerve discharge. *$p<0.001$; #$p<0.05$; +$p<0.01$.

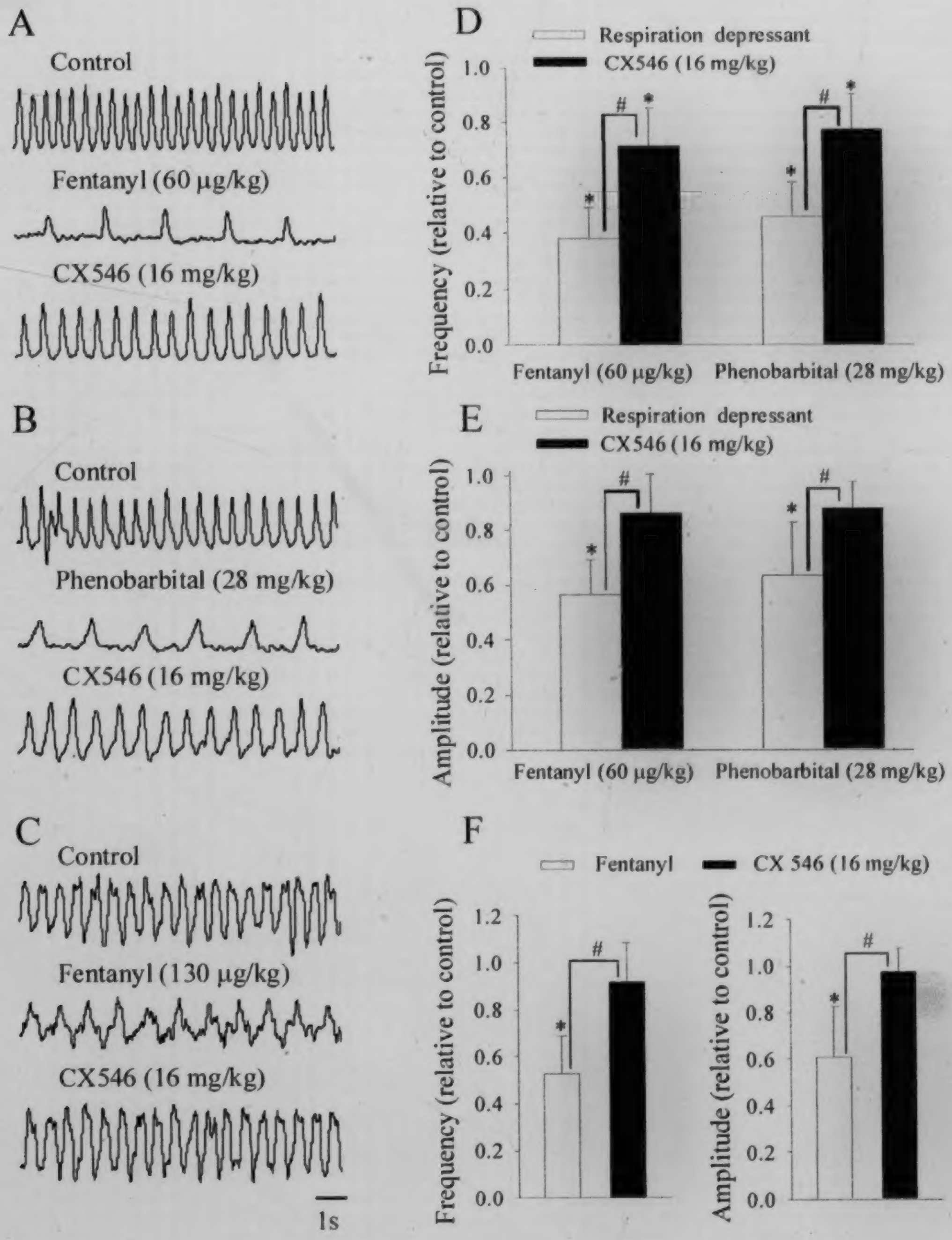
A
Control
Fentanyl (60 μg/kg)
CX546 (16 mg/kg)
B
Control
Phenobarbital (28 mg/kg)
CX546 (16 mg/kg)
C
Control
Fentanyl (130 μg/kg)
CX546 (16 mg/kg)
1s
D
Respiration depressant
CX546 (16 mg/kg)
Frequency (relative to control)
1.0
0.8
0.6
0.4
0.2
0.0
#
*
Fentanyl (60 μg/kg)
Phenobarbital (28 mg/kg)
E
Respiration depressant
CX546 (16 mg/kg)
Amplitude (relative to control)
1.0
0.8
0.6
0.4
0.2
0.0
#
*
Fentanyl (60 μg/kg)
Phenobarbital (28 mg/kg)
F
Fentanyl
CX 546 (16 mg/kg)
Frequency (relative to control)
Amplitude (relative to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
*

Figure 5.5 CX546 counters opioid- and phenobarbital-induced respiratory depression *in vivo*. Plethysmographic recording showing that the inhibitory effect of fentanyl (A) and phenobarbital (B) on the breathing frequency and amplitude in unanesthetized P0 pups is partially countered by CX546. C) Plethysmographic recording from an adult rat showing that, as seen in P0 pups, administration of fentanyl inhibits breathing frequency and amplitude and that this is partially reversed by CX546. D and E) Population data for P0 rat pups showing changes in frequency and amplitude relative to control evoked by fentanyl or phenobarbital both before and after the administration of CX546 (n=4). F) Population data for adult rats showing relative changes in frequency and amplitude evoked by fentanyl alone and after the administration of CX546 (n=4). * indicates significant difference relative to control; # indicates significant difference between values in the presence of the respiratory depressant alone and after subsequent application of CX546. All drugs delivered i.p.

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*CHAPTER VI

AMPAKINE CX717 PROTECTS AGAINST FENTANYL-INDUCED RESPIRATORY DEPRESSION AND LETHAL APNEA

* The manuscript with the results published in this Chapter has been submitted to Anesthesiology. Ren J, Ding X, Greer JJ (2008) Ampakine CX717 protects against fentanyl-induced respiratory depression and lethal apnea. My contribution to this study consisted in the planning and execution of nociception testing and plesthymographic recordings from younger animals. Plesthymographic recordings from adult animals were performed by Ding X.

6.1 INTRODUCTION

Fentanyl is a widely used and effective opiate analgesic for the treatment of acute, postoperative and chronic pain (Swarm et al., 2001). However, fentanyl and other μ-opiate receptor agonists suppress respiratory activity through multiple pathways (Lalley et al., 2008) including direct actions on neurons within the respiratory rhythm-generating center, the preBötzinger complex (preBötC; Greer et al., 1995; Gray et al., 1999). There is a varied susceptibility to fentanyl-induced respiratory depression amongst individuals (Desrosiers, 2006). Predicting which patients are most sensitive, however, is difficult, though, older age, obesity, diseases affecting the respiratory or cardiovascular system and sleep apnea are some of the risk factors for fentanyl-induced respiratory depression. Extended periods of patient controlled analgesia is another major area in which opioid-induced respiratory depression is problematic (Thompson, 2007). Naloxone and related opioid antagonists are currently used to counter respiratory depression in an emergency setting. However, those agents reverse analgesia. Patients are thus experiencing significant pain and clinicians are left trying to find a balance of partial analgesia with manageable respiratory depression. Therefore, there has been a long-felt need to develop a method that allows the analgesic power of opioids to be harnessed without significantly depressing respiratory function.

Previously, we demonstrated that ampakine CX546 (only one commercially available) alleviates the mild fentanyl-induced respiratory depression without interfering the analgesia in rats (Ren et al., 2006). However, whether ampakines could prevent against severe fentanyl-induced severe respiratory depression and fatal apneas was not tested in that study, which is the main purpose of the present study. Further work to evaluate the efficacy of various clinical available members of ampakine family became possible after collaboration between our lab and Cortex Pharmaceuptic InC. One of ampakines, CX717 was tested in this study based on its metabolical stability and proved safety in primate studies and clinical trials for cognitive disorders ((Porrino et al., 2005; Doraiswamy & Xiong, 2006; Wesensten et al., 2007). Here, we demonstrate that the AMPAKINE CX717 prevents severe fentanyl-induced respiratory depression and fatal apneas in rats without interfering with analgesia. Thus, this study has the potential to have an immediate impact in clinical settings.

6.2 MATERIAL AND METHODS

6.2.1 PLETHYSMOGRAPHIC RECORDING METHODS

Measurements from male Sprague-Dawley rats were performed in whole-body, plexi-glass plethysmographs (260 and 2000 ml volume for P17-18 and adult rats, respectively) that had inflow and outflow ports for the continuous delivery of a steady flow of fresh air and removal of expired carbon dioxide.

For intravenous infusion experiments, adult rats (300-370 gms) were anesthetized with isoflurane (3%) in an induction chamber and maintained under isoflurane (2%) anesthesia during tail vein cannulation (P10 size tubing). Animals were then placed within a whole-body plethysmograph that has been further modified to allow exteriorization of the tail for drug infusion (KD Scientific infusion pump). A pulse oximeter (Norin 8600V) was placed on the tail to monitor oxygen saturation levels.

6.2.2 PHARMACOLOGICAL AGENTS

CX717 (provided by Dr. Mark Varney, Cortex Inc.) was dissolved in a 10% 2-hydroxypropyl-β-cyclodextran (HPCD; Sigma) 0.45% saline solution for all experiments (i.p and i.v.). Fentanyl citrate (130 μg/kg; Sandoz) was injected into the left abdomen of P17-18 rats with a 23 gauge needle while the animal remained lightly anesthetized under isoflurane. This gives a stable background respiratory rate similar to that observed in calm or sleeping animals (Colman & Miller 2001; Laferriere et al., 2005). Intravenous drug infusions of varying doses of fentanyl (Dahan et al., 2005; Yassen et al., 2006) or CX717 commenced after approximately 5 minutes of flowing room air through the plethysmograph chamber to remove the residual ambient isoflurane.

6.2.3 NOCICEPTIVE TESTING

Thermal nociception was measured by a modification of a previously reported method (Hargreaves et al., 1988; See Chapter V). Further measure of analgesia in adult rats was performed by examining responses to tail pinching with forceps.

6.2.4 DATA ANALYSIS

Data are expressed as mean ± standard error of the mean (S.E.M.). EC_{50} was calculated using the Hill equation: $E = E_{max}/[1+(EC_{50}/C)^n]$. All statistical comparisons were made against time-matched, vehicle-injected controls. The significance of changes was evaluated by a one-way or two-way ANOVA; $^*p<0.05$ was taken as significant difference.

6.3 RESULTS

6.3.1 INTRAPERITONEAL INJECTIONS OF CX717 IN JUVENILE RATS

The first series of experiments were performed with i.p. administration of fentanyl and CX717 to generate dose-response data of CX717 efficacy. We found that the respiratory depressing effects of i.p. drug delivery of fentanyl to be more consistent in animals aged postnatal day (P)17-18 rats (33- 45 gms) relative to older animals with higher body fat composition. A fentanyl dose of 130 μg/kg (i.p.) induced a marked depression of respiratory frequency within 5-10 minutes in 19 of 24 animals tested. In the remaining 5 rats, there was only a modest (i.e. <30%) decrease in respiratory frequency and those animals were not used for analyses of CX717 effects. Fig 6.1 shows a plethysmographic recording from a P18 rat in response to fentanyl injection. There was a clear decrease in respiratory frequency within 6 minutes and the depression persisted for 45-60 minutes. Our single chamber plethysmograph did not allow for the measurement of absolute tidal volume. However, we could determine that the relative amplitude of the signal was decreased from control levels in all animals after the administration of fentanyl. Fig 6.1B shows the reversal of respiratory frequency depression within 5 minutes of CX717 injection. The amplitude remained depressed relative to control in the presence of CX717. Fig 6.1C and D show the dose-dependent effect of CX717. There were significant alleviations of fentanyl-induced depression of respiratory frequency at concentrations greater than 5 mg/kg CX717 (EC_{50} at 10 min post-CX717 injection = 10.7±0.6 mg/kg). Administration of the vehicle (10% 2-hydroxypropyl-beta-cyclodextran; HPCD) did not cause a significant change in fentanyl-induced depression of respiratory frequency.

We then tested the ability of a pre-administration of CX717 to counter fentanyl-induced respiratory depression. Fig 6.2 shows that 15 mg/kg CX717 (i.p.) was very effective at minimizing fentanyl-induced depression of respiratory frequency when delivered two minutes prior to fentanyl injections. In a further set of experiments, we noted a similar block of fentanyl-induced depression of respiratory frequency when CX717 and fentanyl were co-administered in a cocktail (data not shown). Thus, the two compounds have a similar onset of action time course.

CX717 did not significantly alter fentanyl-induced analgesia. The mean response time of paw withdrawal to a thermal stimulation in control, CX717 alone, fentanyl, and fentanly-CX717 conditions were 6.7 ± 0.9 sec, 6.6 ± 0.8 sec, > 20 sec, > 20 sec, respectively (n=5-7 each group). Further, the level of sedation induced by fentanyl was not altered by CX717. With the relatively high dose of fentanyl used, all animals showed clear muscle rigidity, as reported in previous studies (Scamman, 1983; Jerussi et al., 1987; Benvenga, 1992).

6.3.2 INTRAVENOUS ADMINISTRATION OF CX717 IN ADULT RATS

Having determined the dose-response characteristics of CX717, we performed a further study using older rats (300-370 gms) and evaluated the effects of i.v. infusions. Administration of CX717 pre-, concomitant with or post-fentanyl were performed to determine their efficacy in countering respiratory depression. In the first paradigm, 60 μg/kg fentanyl (i.v.) was delivered over a 20 minute infusion period (Fig 6.3). This caused a marked suppression of respiratory frequency (>50% of control) that lasted for the duration of fentanyl infusion. In approximately 40% of those rats (e.g. Fig 6.3A), there was a partial rebound in respiratory frequency following an initial slowing of breathing frequency. This partial rebound was typically followed by further depression of respiratory frequency as the fentanyl infusion continued. In the remaining 60% of rats, the level of respiratory depression was more constant (Fig 6.4A). Injection of CX717 (15 mg/kg i.v.; n=7) six minutes after the fentanyl administration caused an increase in respiratory frequency within 1 minute that lasted beyond the duration of the fentanyl infusion (Fig 6.3B & C). Further, oxygen saturation levels 10 minutes after the fentanyl injection were elevated from 59.6 ± 2.4% (vehicle group, n=9) to 80.1 ± 4.6% (n=7) in the presence of CX717 administration.

We then tested whether CX717 (15 mg/kg, i.v.) administered prior to fentanyl (60 μg/kg; 20 minute infusion) would prevent the fentanyl-induced respiratory depression (n=10). Fig 6.4B shows that pretreatment of CX717 significantly prevented fentanyl-induced depression of respiratory frequency. Note that the initial increase in respiratory frequency after CX717 infusion was due to the countering of the minor residual respiratory depression resulting from isoflurane administration during the tail vein cannulation. Population data are summarized in Fig 6.4C. The oxygen saturation levels 10 minutes after fentanyl were elevated from 64.6 ± 4% (n=9) to 76.7 ± 2.4% (n=10) with pre-administration of CX717. Infusion of the vehicle (10% HPCD) did not cause a significant change in fentanyl-induced depression of respiratory frequency or oxygen saturation levels.

Fig 6.5A shows data from an experiment in which 80 μg/kg fentanyl (n=5) was infused rapidly. This administration protocol induced profound apnea that is typically lethal. Injection of CX717 (15 mg/kg i.v.; n=5) 30-60 s after the onset of fentanyl-induced apnea, led to a marked rebound of respiratory frequency in all 5 animals tested (Fig 6.5B). Pre-administration of 15 mg/kg CX717 (n=5) immediately prior to delivering the fentanyl bolus minimized the period of apnea and all of the animals survived (Fig 6.5C). Population data are summarized in Fig 6.5D.

During the thermal nociceptive testing, the mean response time of paw withdrawal in control (10% HPCD), CX717 (15 mg/kg, i.v.), fentanyl (60 μg/kg, i.v.), and fentanly-CX717 conditions were 6.1 ± 1.1 sec, 6.3 ± 1.4 sec, > 20 sec, > 20 sec, respectively (n=4-5 each group). Further, CX717 did not induce any notable change in sedation, response to tail clamping or loss of righting reflex.

The body temperature decreased by 1.2 ± 0.1°C and 1.8 ± 0.2°C after 20 and 50 min, respectively, in response to a 20 minute infusion of 60 μg/kg fentanyl. This decrease in body temperature was 1.0 ± 0.1°C at both time points in the presence of CX717. Presumably, the increased respiratory frequency in the presence of CX717 helped maintain body temperature. Varying degrees of fentanyl-induced muscle rigidity were evident in all animals tested.

6.4 DISCUSSION

Data from studies using *in vitro* and *in vivo* models support the hypothesis that the basic rhythm underlying breathing arises from a specific region of the ventrolateral medulla, the preBötC (Smith et al., 1991; Solomon et al., 1999; Gray et al., 2001). The precise mechanism underlying the generation of rhythmogenesis within the preBötC has not been resolved (Feldman & Del Negro, 2006). However, the neurotransmitter glutamate, acting via AMPARs, is a critical component for the generation of respiratory rhythm within the preBötC (Greer et al., 1991; Funk et al., 1993; Pace et al., 2007). Fentanyl induces depression of respiratory frequency in part by direct actions at μ-opiate receptors expressed on neurons within the preBötC (Greer et al., 1995; Gray et al., 1999). Thus, mechanistically, a significant component of the CX717 effect can be explained by accentuation of AMPA receptor-mediated glutamatergic excitation that will counteract the μ-opioid receptor-mediated suppression of preBötC neuronal excitability. In addition, CX717 may also be acting via additional neuronal populations in which AMPA-mediated conductances play an important role. This would include neurons of the nucleus of the solitary tract, retrotrapezoid nucleus, raphe complex and pontine respiratory nuclei. All of these provide modulatory synaptic drive that regulates preBötC rhythmogenesis (Feldman & Del Negro, 2006). In clinical studies, CX717 has shown efficacy in reducing inattentiveness and hyperactivity in adults with ADHD (Adler et al., 2006). The dose in this study was 800 mg twice-a-day, or approximately 11 mg/kg twice-a-day. Thus, the doses required to reverse opiate-induced respiratory depression in rats are consistent with efficacious doses in previous clinical studies.

The fentanyl-induced suppression of plethysmographic pressure change amplitude was not significantly reversed by CX717. This is despite the fact that AMPA receptor activation plays a role in excitatory inspiratory drive to cranial and spinal motoneurons (Greer et al., 1991; Funk et al., 1993). In contrast, recordings from *in vitro* brainstem-spinal cord and medullary slice preparations show that CX717 produces a clear reversal of opioid induced suppression of inspiratory motoneuron population discharge amplitude (Lenal et al., 2007). The discrepancy likely results from the fact that fentanyl, particularly at the high doses used in this study, cause changes in upper airway and ribcage compliance *in vivo* (Scamman, 1983). This 'fentanyl-induced stiffness' is thought to be, in part, mediated via alpha-2 adrenergic receptors (Jerussi et al., 1987; Benvenga et al., 1992). Ampakines would not directly affect that aspect of fentanyl-induced properties. Lower doses of fentanyl used in humans typically alter respiratory frequency to a greater degree relative to amplitude (Ferguson & Drummond, 2006) and thus should be very amenable to reversal by CX717. Further, unlike earlier studies of AMPAR modulators (Funk et al., 1995; Ren et al., 2006), CX717 readily crosses the blood-brain barrier, is metabolically stable and does not produce significant unwarranted side-effects. Hence, CX717 is an agent that enhances the safety of using opiate drugs while preserving the analgesic effects. This advancement could significantly improve pain management in a variety of clinical settings.

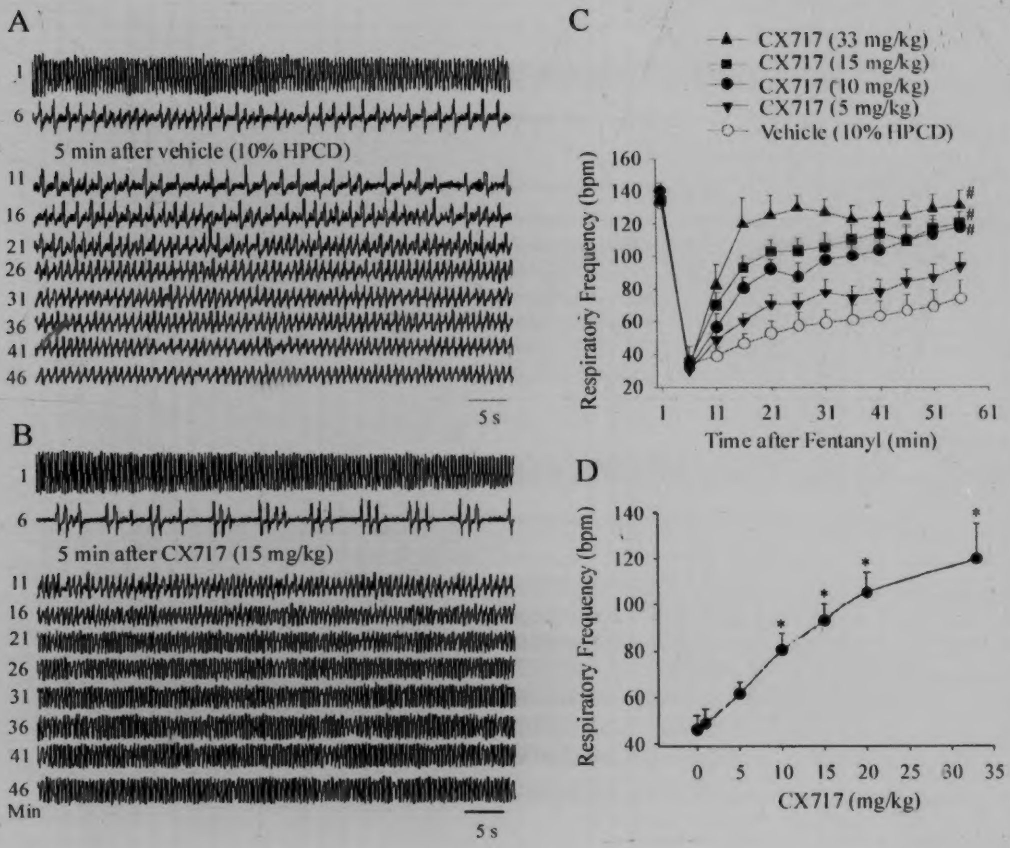
A
1
6
5 min after vehicle (10% HPCD)
11
16
21
26
31
36
41
46
5 s
B
1
6
5 min after CX717 (15 mg/kg)
11
16
21
26
31
36
41
46
Min
5 s
C
CX717 (33 mg/kg)
CX717 (15 mg/kg)
CX717 (10 mg/kg)
CX717 (5 mg/kg)
Vehicle (10% HPCD)
Respiratory Frequency (bpm)
160
140
120
100
80
60
40
20
1
11
21
31
41
51
61
Time after Fentanyl (min)
#
#
#
D
Respiratory Frequency (bpm)
140
120
100
80
60
40
0
5
10
15
20
25
30
35
CX717 (mg/kg)
*
*
*
*

Figure 6.1 CX717 (i.p.) alleviates respiratory depression induced by fentanyl (i.p.) in postnatal day (P)17-18 animals. A) In this, and remaining figures, the traces shown are whole-body plethysmographic measurements of the frequency and relative depth of breathing in unrestrained rats. Numbers on the left of traces refer to minutes after injection of 130 μg/kg fentanyl. Injection of the vehicle HPCD 6 minutes after fentanyl administration had no effect on the depression of respiratory frequency. B) In another P18 rat, the injection of CX717 (15 mg/kg i.p.) 6 minutes after fentanyl administration alleviated a significant component of the depression of respiratory frequency. C) Dose-response data showing the relative frequency of breathing following injection of vehicle or increasing doses of CX717 six minutes after the injection of fentanyl; # $p < 0.05$ indicates significance relative to the vehicle control group (n=9-13 animals for each dose). D) Average respiratory frequency 10 minutes after the injection of various doses of CX717. Significant changes of respiratory frequency relative to vehicle control were achieved at >5 mg/kg CX717 (i.p.) with the EC50=10.7 ± 0.6 mg/kg. * $p < 0.05$ indicates a significant difference relative to vehicle control respiratory frequency.

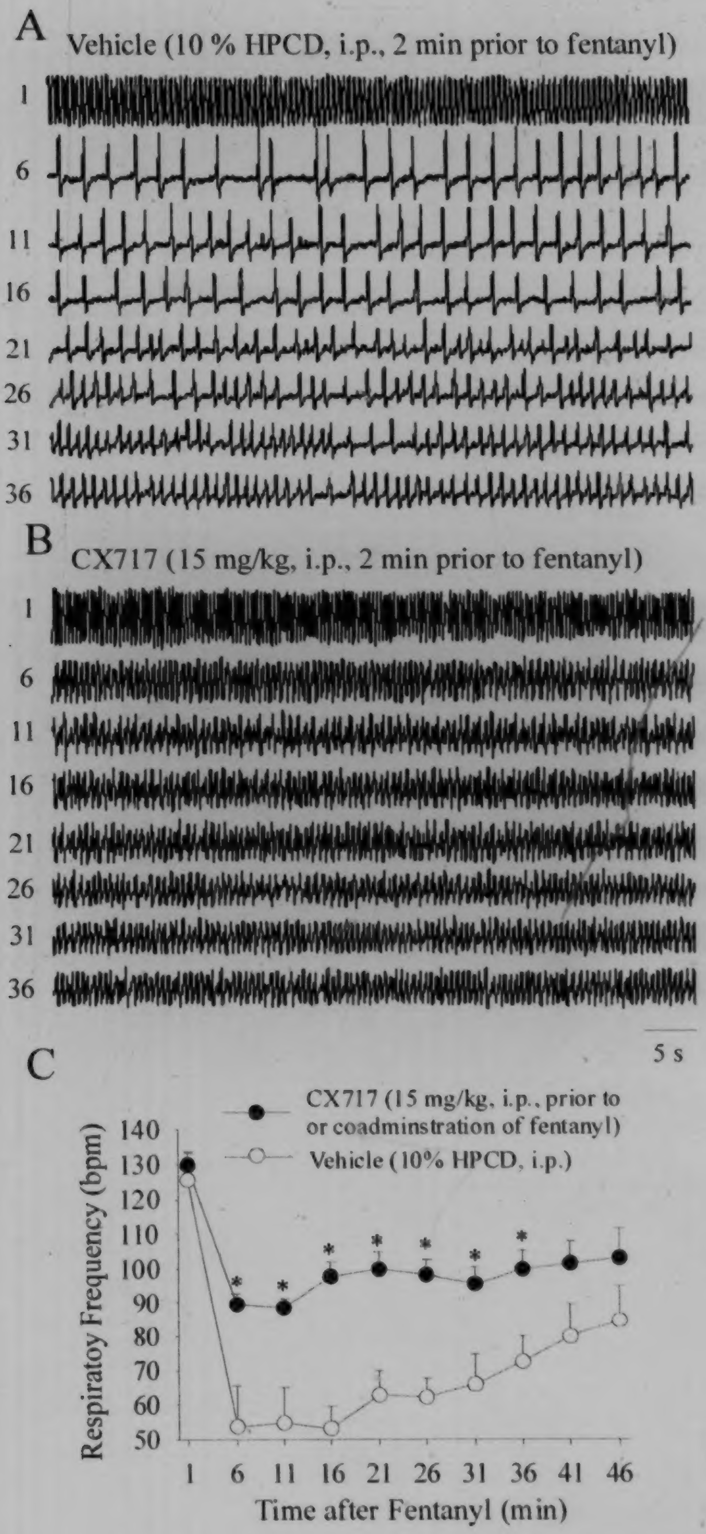
A
Vehicle (10 % HPCD, i.p., 2 min prior to fentanyl)
1
6
11
16
21
26
31
36
B
CX717 (15 mg/kg, i.p., 2 min prior to fentanyl)
1
6
11
16
21
26
31
36
5 s
C
CX717 (15 mg/kg, i.p., prior to or coadminstration of fentanyl)
Vehicle (10% HPCD, i.p.)
Respiratoy Frequency (bpm)
140
130
120
110
100
90
80
70
60
50
*
1
6
11
16
21
26
31
36
41
46
Time after Fentanyl (min)

Figure 6.2 Pre-administration of CX717 (i.p.) prevents respiratory depression induced by fentanyl in P17-18 rats. A) Pre-administration of the vehicle HPCD solution (i.p.) had no effect on the fentanyl-induced suppression of respiratory frequency in a P17 rat. B) In another P17 rat, administration of CX717 (15 mg/kg, i.p.) two minutes prior to the fentanyl administration markedly alleviated depression of respiratory frequency. C). Population data showing the time course of changes of respiratory frequency in response to fentanyl administration following pre-administration of vehicle or CX717. * *p*< 0.05 indicates a significant difference relative to vehicle control respiratory frequency (n=9 for each group).

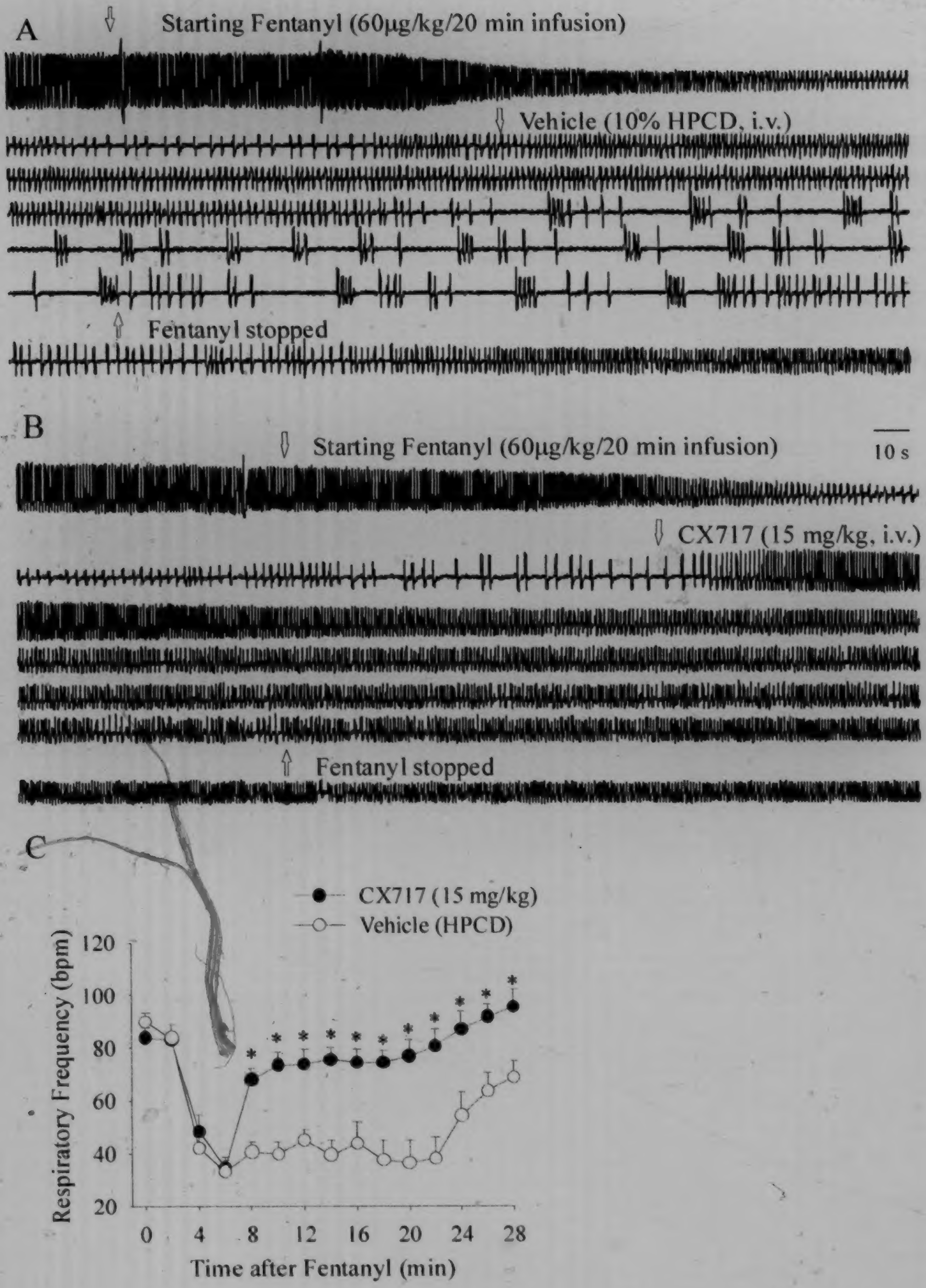
A
Starting Fentanyl (60μg/kg/20 min infusion)
Vehicle (10% HPCD, i.v.)
Fentanyl stopped
B
Starting Fentanyl (60μg/kg/20 min infusion)
10 s
CX717 (15 mg/kg, i.v.)
Fentanyl stopped
C
CX717 (15 mg/kg)
Vehicle (HPCD)
Respiratory Frequency (bpm)
120
100
80
60
40
20
0
4
8
12
16
20
24
28
Time after Fentanyl (min)
*

Figure 6.3 CX717 (i.v) counters respiratory depression induced by fentanyl (i.v.) in adult rats. A) Delivery of 60 μg/kg fentanyl (i.v.) over a 20 minute infusion period caused an initial marked depression of respiratory frequency. In this particular rat, there was a partial rebound of frequency and then a subsequent further decrease of respiratory rate. Depression of respiratory frequency persisted after i.p. administration of vehicle HPCD. B) In another adult rat, infusion of CX717 (15 mg/kg) approximately six minutes after the commencement of the fentanyl infusion caused a rapid and marked alleviation of respiratory frequency depression. C) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration with the subsequent administration of vehicle or CX717. * $p < 0.05$ indicates a significant difference relative to vehicle control respiratory frequency (n=7-9 for each group).

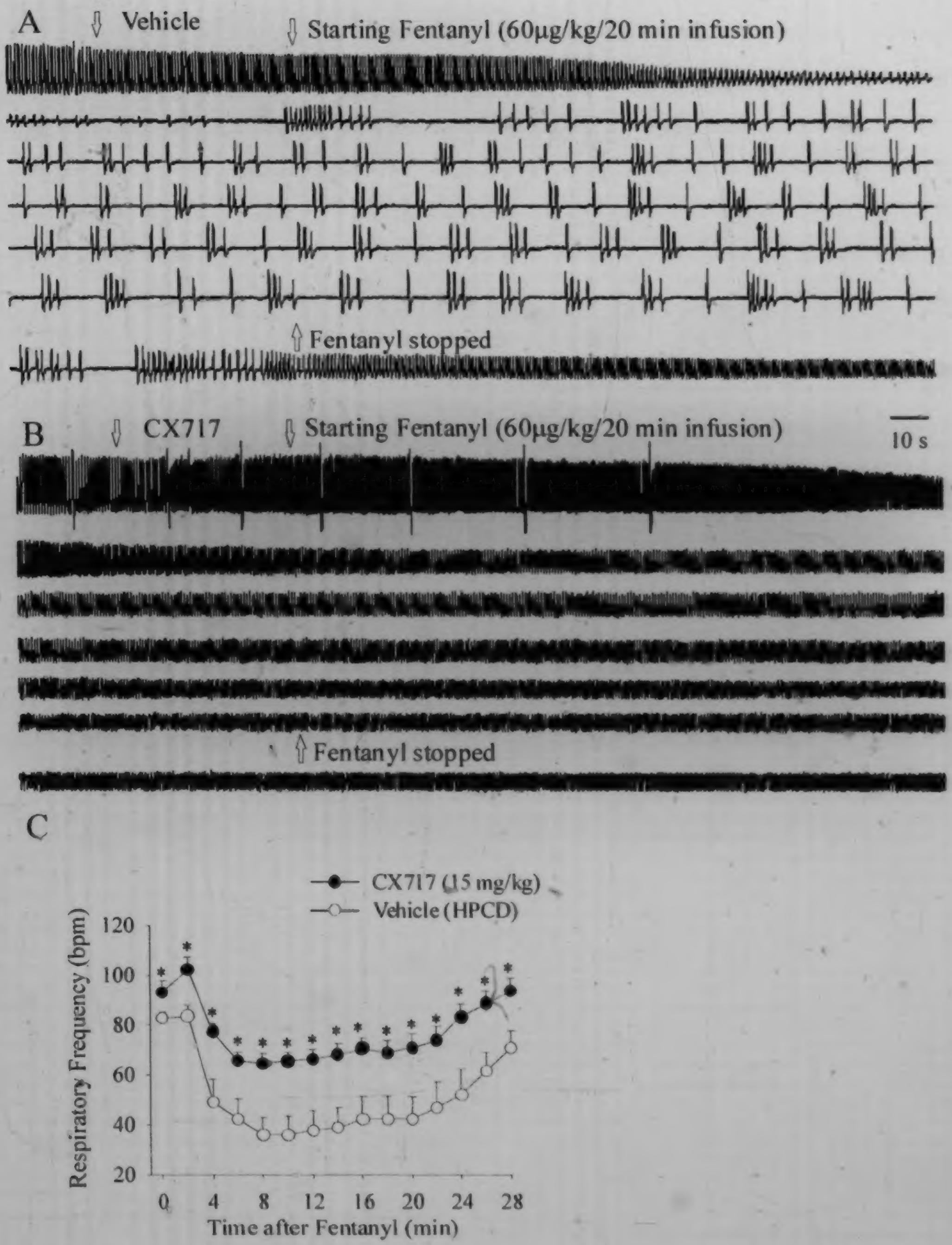
A
Vehicle
Starting Fentanyl (60μg/kg/20 min infusion)
Fentanyl stopped
B
CX717
Starting Fentanyl (60μg/kg/20 min infusion)
10 s
Fentanyl stopped
C
CX717 (15 mg/kg)
Vehicle (HPCD)
Respiratory Frequency (bpm)
120
100
80
60
40
20
0
4
8
12
16
20
24
28
Time after Fentanyl (min)
*

Figure 6.4 Pre-administration of CX717 (i.v.) prevents respiratory depression induced by fentanyl (i.v.) in adult rats. A) The administration of 60 μg/kg i.v. fentanyl delivered over a 20 minute infusion period produced suppression of respiratory frequency that was not affected by the pre-administration of the vehicle HPCD (i.v). B) In another adult rat, pre-administration of CX717 (15 mg/kg, i.v.) markedly attenuated the fentanyl-induced depression of respiratory frequency. C) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration with the pre-administration of vehicle or CX717. * $p < 0.05$ indicates a significant difference relative to vehicle control respiratory frequency (n=9-10 for each group).

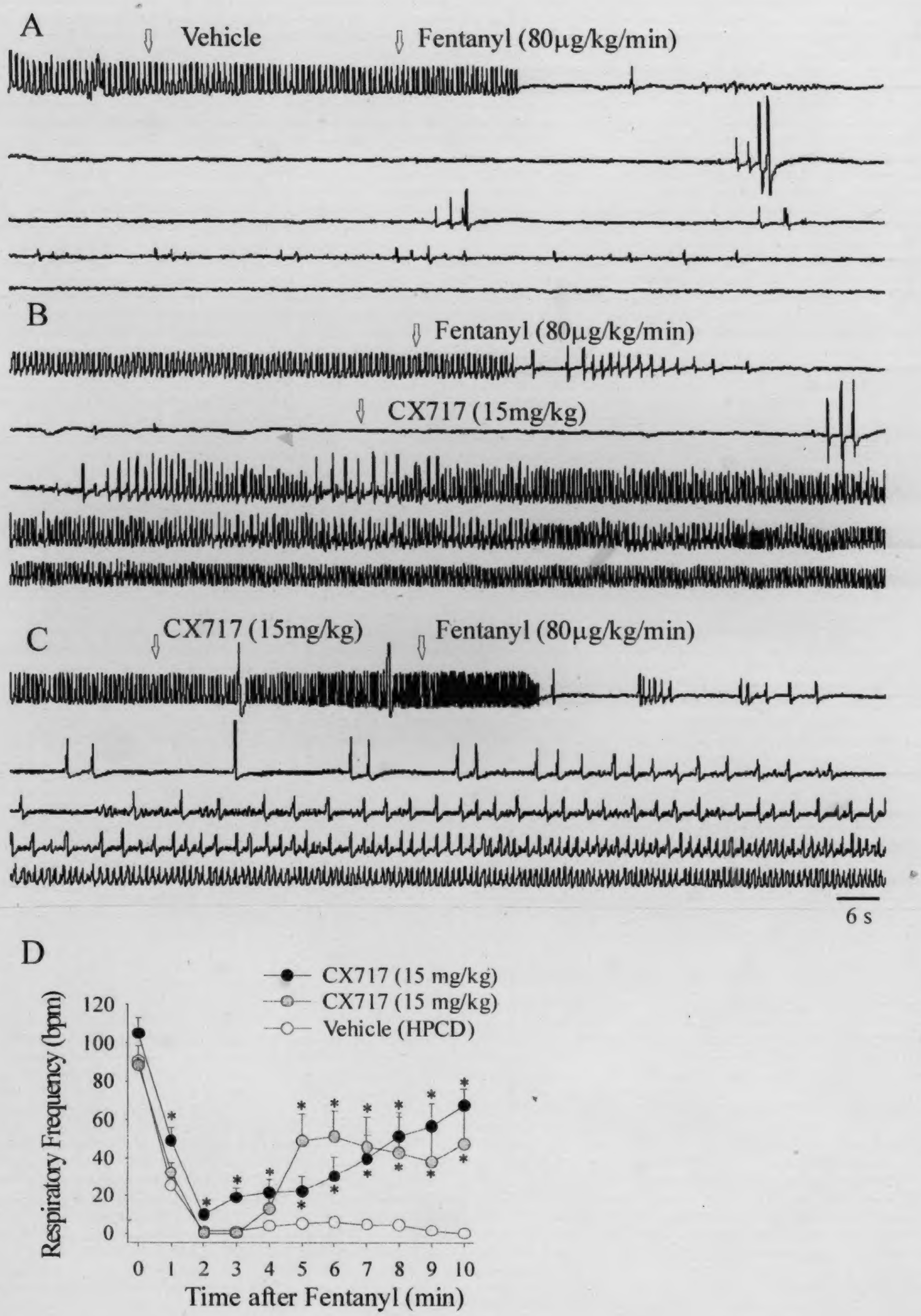
A
Vehicle
Fentanyl (80µg/kg/min)
B
Fentanyl (80µg/kg/min)
CX717 (15mg/kg)
C
CX717 (15mg/kg)
Fentanyl (80µg/kg/min)
6 s
D
CX717 (15 mg/kg)
CX717 (15 mg/kg)
Vehicle (HPCD)
Respiratory Frequency (bpm)
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8 9 10
Time after Fentanyl (min)
*

Figure 6.5 Administration of CX717 (15 mg/kg i.p.) reverses/prevents fentanyl-induced lethal apnea in adult rats. A) Administration of a high dose of fentanyl (80 μg/kg) over a short period (1 minute) induced a profound, lethal respiratory depression. Vehicle (HPCD) injection prior to fentanyl administration had no significant effects on depression of respiratory frequency. B) Administration of CX717 (15 mg/kg) within one minute after the fentanyl infusion reduced the period of apnea and induced the recovery of respiratory rhythm. C) Pre-administration of CX717 (15 mg/kg, i.p.) prior to the fentanyl infusion reduced the severity of fentanyl-induced apnea and depression of respiratory frequency and prevented death. D) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration with pre- or post-administration of vehicle or CX717. *$p < 0.05$ indicates a significant difference relative to vehicle control respiratory frequency (n=5 for each group).

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CHAPTER VII

GENERAL DISCUSSION

The primary goals of the thesis were to advance our understanding of key neurochemical modulators responsible for regulating respiratory rhythmogenesis and motoneuron drive in physiological and pathophysiological conditions and to develop a potential drug therapy to alleviate central respiratory depression. The first project was to determine the actions of chloride-mediated conductances via glycine and $GABA_A$ receptors on respiratory rhythmogenesis in perinatal rats (Chapter II). The second project was to test whether $GABA_A$ receptor-mediated modulation of respiration is further influenced by neurosteroids (Chapter III). The third project tested the hypothesis that abnormal neuronal activity within the putative respiratory rhythm-generating center was responsible for the respiratory dysfunction in the *necdin*-deficient mouse, an animal model for the neurodevelopmental disorder PWS (Chapter IV). An ultimate purpose of our concerted efforts in the studies of neurochemical modulation of central respiratory rhythm is to develop a potential novel and clinically relevant pharmacological means of alleviating respiratory depression. Thus, in the final two projects (Chapter V and Chapter VI), I tested the hypothesis that the ampakine class of drugs can counter respiratory depression associated with opioid analgesics, anesthetics and weak endogenous drive.

7.1 NEUROCHEMICAL MODULATION OF RESPIRATORY CONTROL

One of the primary goals of respiratory research is to understand the neurotransmitter systems responsible for maintaining and modulating respiratory rhythmogenesis and motoneuron drive. Non-NMDA type glutamate receptors (primarily AMPA receptors) constitute the primary excitatory synaptic connectivity between respiratory neurons (Greer et al., 1991; Funk et al., 1993, 1995; Thoby-Brisson et al., 2005). Further, respiratory activity is modulated by a diversity of neuromodulators such as serotonin, norepinephrine, or opioids (Bonham 1995; Ballanyi et al., 1999; Ballanyi 2004). The knowledge of neurochemical control of respiration will provide us with an insight into the pathogenesis and pharmacotherapy of the breathing disorders (Peña & García, 2006).

In the first project, we systematically investigated the effects of chloride-mediated conductances via the activation of $GABA_A$ and glycine receptors on the generation of respiratory rhythm in newborn rats and prenatally from the time of inception of fetal breathing movements (FBM). The rationale for this study was based on the following. First, as principal mediators of fast inhibitory transmission in the mammalian CNS, the neurotransmitters GABA and glycine strongly modulates respiratory rhythm (Johnson et al., 1996; Shao & Feldman, 1997; Brockhaus & Ballanyi, 1998; Ritter & Zhang, 2000). However, studies of the function of GABA and glycine in respiratory control during the neonatal period had yielded contradictory results. Whether $GABA_A$- and glycine receptor-mediated actions are depolarizing/hyperpolarizing resulting in stimulation/ depression of respiratory frequency in neonates is unclear (Brockhaus & Ballanyi, 1998; Ritter & Zhang, 2000). Second, there had been no systematic study regarding the modulation of GABA and glycine on respiratory rhythm during the prenatal period.

Our initial extracellular recordings from the brainstem-spinal cord preparation revealed that the transition from excitatory to inhibitory effects on respiratory rhythmogenesis occurs at approximately embryonic day 19 (Ren & Greer, 2006a). The timing was consistent with our non-respiratory rhythm data over development in spinal cord preparation (Ren & Greer, 2003a, Ren et al., 2006c). We then made recordings of nerve roots and/or neuronal population discharge from medullary slice preparations; unexpected excitatory effects of $GABA_A$- and glycine receptor-mediated actions were observed. The seemingly chloride-mediated contradictory actions between the two types of *in vitro* preparation were examined to differentiate among three possibilities: (1) the difference of preparations per se, (2) direct verse indirect effects of agonists, and (3) different extracellular potassium concentration $[K^+]_o$) used in these two types of preparation. The fact that chloride-mediated contradictory actions persisted in the presence of bath applied antagonists to most key neurotransmitter-mediated receptors and after sequential serial section of preparations suggested that both inhibitory and excitatory actions are direct, and not due to the difference of two types of *in vitro* preparations per se. Further, alterations in $[K^+]_o$ accordingly changed the direction of chloride-mediated actions in both two types of preparations, specifically, 3 mM $[K^+]_o$-mediated inhibitory while 9 mM $[K^+]_o$-mediated excitatory effects. I then performed gramicidin perforated patch recordings (to keep intracellular chloride ion intact, Kyrozis & Reichling, 1995) from respiratory neurons in medullary slice preparations over perinatal development to explore the cellular mechanisms underlying the effects of chloride-mediated conductances in different $[K^+]_o$ (i.e. 3 mM verse 9 mM $[K^+]_o$). Further, *in vivo* plethysmographic recordings from unanesthetized pups were performed. Through the systematic study with a combination of *in vivo* and *in vitro* preparations, we concluded that by birth, GABA, glycine, and taurine all induce a hyperpolarization of the membrane potential in respiratory medullary neurons and a suppression of respiratory frequency. With respect to inhibitory synaptic processes, it is important to note that the transmembrane chloride gradient has a developmental change that determines the function of GABA and glycine-mediated chloride conductances during developmental stage. Further, I demonstrated that the age-dependant change in the actions of chloride-mediated conductances is functionally regulated by the development of chloride co-transporters (KCC2 and NKCC1). This is consistent with a high expression of NKCC1 (pumping Cl^- into the cytoplasm) in immature neurons, and increased expression of KCC2 (extruding Cl^- from the cytoplasm) and decreased expression of NKCC1 in mature neurons over development (Kaila, 1994; Rohrbough & Spitzer, 1996; Rivera et al., 1999). Interestingly, we also found that the function of KCC2 chloride co-transporter is strongly modulated by $[K^+]_o$, confirming previous findings that KCC2 could extrude or accumulate Cl^- depending on $[K^+]_o$ (Payne, 1997; DeFazio et al., 2000). This must be considered when analyzing actions of some neuromodulators using *in vitro* preparations perfused with elevated $[K^+]_o$ solution. Indeed, a recent study demonstrated that elevated $[K^+]_o$ contributed to the decreased sensitivity to neuromodulator-activated signalling pathways in rhythmic medullary slice preparations (Ruangkittisakul et al., 2006).

There is also a developmental change in the sensitivity of the respiratory network of rodents to other neuromodulators during the perinatal period. For example, both TRH and 5-HT exert a much stronger stimulatory effect on respiratory frequency in prenatal relative to postnatal rat *in vitro* preparations (DiPasquale et al., 1996; Funk et al., 2004; Greer et al., 1996; Onimaru et al., 1998). Similar to $GABA_A$ and glycine receptor mediated age-dependent effects, the action of prostaglandin on respiratory rhythm switches from an excitatory to inhibitory action at E20 rat (Ballanyi, 2004; Ballanyi et al., 1999). The functional importance is unclear regarding the developmental change. However, in the early stage of development, the depolarization of respiratory neurons by these modulators may regulate synaptic transmission, promote neuronal development (Yuste and Katz, 1991; Ben Ari et al., 1997; Ziskind-Conhaim, 1998), and facilitate the functional maturity of ventral respiratory group neurons (DiPasquale et al., 1996). The activation of AMPA type glutamate receptors provides a primary conditioning drive to maintain the oscillator state of the key neurons within the respiratory rhythm generating center and mediates an excitatory drive to motoneurons innervating respiratory-related muscles both in postnatal (Greer et al., 1991; Funk et al., 1993, 1995) and prenatal (Thoby-Brisson et al., 2005) rodent models. Our previous study of non-respiratory rhythm from E17 rat spinal cord preparations demonstrated that it was mediated by a combination of activation of $GABA_A$, glycine, and non-NMDA receptors (Ren & Greer, 2003a). Therefore, administration of antagonists of $GABA_A$ and glycine receptors may reveal a similar role of GABA, glycine and perhaps taurine in the generation and transmission of respiratory drive in the first day of respiratory rhythmogenesis from *in vitro* brainstem-spinal cord and medullary slice preparations (E17).

In late gestation and the newborn period, neuromodulators including GABA and glycine will regulate respiratory frequency and shape the respiratory pattern (Brockhaus & Ballanyi, 1998; Ballanyi et al., 1999). Taurine is in high concentrations within the medulla during the perinatal period (Sturman, 1993), and levels increase within the ventrolateral medulla during hypoxic-induced depression of newborn breathing (Hoop et al., 1999). These and the fact that it reduces respiratory frequency when administered within the cerebral ventricles (Holtman et al., 1983) or by bath application in the *in vitro* brainstem-spinal cord and medullary slice preparations (Ren & Greer, 2006b), suggest that it may contribute to hypoxic-induced respiratory depression. Future experiments examining for increased taurine, GABA, or glycine release both *in vitro* and *in vivo* in response to hypoxia would provide direct evidence for the involvement of these neurotransmitters in the hypoxic-induced respiratory depression in the perinatal period.

Subsequently, I tested the hypothesis that $GABA_A$ receptor-mediated modulation of perinatal respiration is influenced by the presence of neurosteroids. I examined the actions of allopregnanolone and DHEAS on the spontaneous respiratory drive generated by *in vitro* and *in vivo* models. These two neurosteroids are prevalent in the perinatal CNS and have pronounced modulatory effects on $GABA_A$ receptor function (Compagnone et al., 1995; Nguyen et al., 2003). I demonstrated that the efficacy of $GABA_A$ receptor-mediated modulation of respiratory membrane potential and rhythmogenesis is markedly enhanced by allopregnanolone and depressed by DHEAS. These observations suggest that the modulation of breathing via $GABA_A$ receptor activation is determined by the overall balance of negative and positive neurosteroid modulators within respiratory nuclei (Ren & Greer, 2006b). This adds a level of complexity that must be considered when examining the depression of breathing in mammals associated with various behavioural states and pathogenic conditions.

Previous studies (Hirst et al., 2006, 2008) have demonstrated that neurosteroids such as allopregnanolone are synthesized in the fetal brain either from cholesterol or from circulating precursors derived from the placenta. The concentrations of allopregnanolone are remarkably high in the fetal brain and rise further in response to acute hypoxic stress, induced by constriction of the umbilical cord. This response may result from the increased 5α-reductase and cytochrome P-450(SCC) expression in the brain. These observations suggested that the rise in neurosteroid concentrations in response to acute hypoxia may represent an endogenous protective mechanism that reduces excitotoxicity following hypoxic stress in the developing brain. At birth, the allopregnanolone concentration in the brain falls markedly, probably due to the loss of placental precursors; however, stressors, including hypoxia and endotoxin-induced inflammation, raise allopregnanolone concentrations in the newborn brain. This may protect the newborn brain from hypoxia-induced damage. However, the rise in allopregnanolone concentrations was also associated with increased sleep, which may depress arousal and the respiratory rhythmogenesis. These may contribute to the risk of SIDS. Thus, we proposed that the acute hypoxic stress-induced elevated allopregnanolone (by binding to $GABA_A$ receptors) may protect against the hypoxia-induced damage by overall suppression of fetal CNS activity, while simultaneously post a life-threatening risk due to suppression of respiratory rhythmogenesis and arousal from the hypoxic condition. After birth, the decline in allopregnanolone levels may result in greater vulnerability to brain injury in neonates, whereas, decreased efficacy of inhibitory actions of allopregnanolone on respiratory rhythm may provide a compensated protection. Thus, further neurosteroid studies should be able to identify the timing and spatial distribution of neurosteroid expression (e.g. 5α-reductase), and whether neurosteroids are released or functionally involved (e.g. pretreatment with 5α-reductase inhibitor, finasteride) during episodic FBM or hypoxia.

7.2 CENTRAL RESPIRATORY DISORDERS IN GENETIC MOUSE MODELS

Genetic manipulations provide the opportunity to inactivate, eliminate or alter specific neurones to dissect and understand central rhythm and pattern generating networks at the molecular, cellular, network and physiological levels (Kiehn & Kullander, 2004; Goulding & Pfaff, 2005). A combination of *in vivo* and *in vitro* experiments is used to examine mutant mouse models with breathing abnormalities (Blanchi & Sieweke, 2005; Gaultier & Gallego 2008; Greer 2008). *In vivo* plethysmography is used to compare wild-type and mutant mouse breathing parameters in room air, hypoxic, and hypercapnic conditions. A defect *in vivo* can reflect dysfunctions at many points in the respiratory control system (e.g., chemoreception, afferent signalling, motoneurons, muscle, central neural networks), which can be further delineated by *in vitro* and *in situ* preparations. Specifically, to determine if there is a defect in respiratory rhythmogenesis per se, brainstem-spinal cord and medullary slice *in vitro* preparations are used to measure the basic respiratory drive within the preBötC and to motoneuron pools. In addition, one can determine if a defective rhythm can be normalized by the addition of appropriate neurochemical drive. Parallel anatomical studies reveal structural defects. These strategies have been successfully employed to examine models with defects in transcription factors hypothesized to be important for brain stem development (Blanchi & Sieweke, 2005; Pagliardini et al., 2008) as well as genetic mouse models of RTT (Viemari et al., 2005; Ogier et al., 2007), PWS (Ren et al., 2003b; Pagliardini et al., 2005; Zanella et al., 2008), and CCHS (Dubreuil et al., 2008).

Necdin is one of a cluster of genes deleted in the neurodevelopmental disorder PWS. *Ndn*^{tm2Stw} mice die in the neonatal period of apparent respiratory insufficiency. We demonstrated that the defect can be explained by abnormal neuronal activity within the preBötC, a putative respiratory rhythm-generating center. Specifically, the rhythm is unstable with prolonged periods of depression of respiratory rhythmogenesis. These observations suggest that the developing respiratory center is particularly sensitive to loss of *necdin* and may reflect abnormalities of respiratory rhythm-generating neurons or conditioning neuromodulatory drive. Further, *in vitro* studies determined if the unstable respiratory rhythm in the *Ndn*^{tm2Stw} mice can be normalized by neuromodulators. We found that exogenous application of excitatory neuromodulators alleviated the long periods of slow respiratory rhythms and apnea, but some instability of rhythmogenesis persisted. Our interpretation is that the exogenous application of neuromodulators overcame much of the deficit resulting from the abnormalities in medullary structures that normally provide conditioning drive to the preBötC. These observations were consistent with the findings of (1) normal gross structure of the preBötC in *necdin*-deficient mice, as shown by the presence of NK1R- and somatostatin-immunopositive neurons within the preBötC, and (2) clear anatomical abnormalities in surrounding medullary structures that provide conditioning synaptic input to respiratory rhythmogenic neurons (Pagliardini et al., 2005). The functional defect could reflect changes in neuronal properties or abnormalities in the preBötC network connectivity due to problems with axon guidance and fasciculation. It is noteworthy that many of the mutant mouse models have multiple abnormalities in structures that modulate preBötC function (Gaultier & Gallego 2008; Greer 2008). Thus, it is likely a combination of defects in the conditioning drive that causes the respiratory phenotype in many of these models rather than a specific defect within the pre-BötC or one single respiratory nucleus.

Overall, an increasing number of genetic mutations in mouse models are associated with hypoventilation, which has been linked to abnormalities of the central control of respiratory rhythmogenesis (Blanchi & Sieweke, 2005; Pagliardini et al., 2005, 2008). Data from these models provide insights into the transcriptional control mechanisms underlying respiratory network development and breathing disorders in the perinate, including CCHS (Dubreuil et al., 2008), RTT (Viemari et al., 2005; Ogier et al., 2007), and PWS (Ren et al. 2003b; Pagliardini et al. 2005). The fact that the defective rhythm could be partially normalized by the addition of appropriate neurochemical drive in some of these genetic mouse models with central respiratory disorders (Pagliardini et al., 2005; Viemari et al., 2005; Ogier et al., 2007), provides an opportunity of developing pharmacological approaches to alleviate these devastating problems.

The strain of Ndn^{tm2Stw} mouse that we analyzed dies shortly after birth (most within 1 hour). The multiple abnormalities in structures that modulate preBötC function and perhaps the preBötC per se contributed to the central respiratory defect of brainstem development (Pagliardini et al., 2005). Using a different strain of *necdin*-deficient mice with some survivors living up to adulthood, Zanella et al. (2008) proposed the defects in serotonergic modulation of respiratory network are responsible for the central respiratory dysfunction including the SA observed in the PWS patients and *necdin*-deficient mice. However, the behavior-related modulation of respiratory rhythmogenesis has not yet been analyzed, although some features (i.e. behavior, mental problems and earlier obesity) of the PWS suggest that behavioral abnormalities may be associated with the defect in the breathing control. Very recently, our colleague at the Univesity of Alberta, Dr. Rachel Wevrick, has successfully generated a new colony of *necdin*-deficient mice that survive to adulthood. Significant respiratory dysfunctions including more pronounced apneas were observed from *in vivo* plethysmographical recordings of these *necdin*-deficient mice at the age of ~ three months old (our preliminary data). It will be interesting to use this new *necdin* colony to systematically analyze the time course (prior to or post obesity) and the cause (i.e. stress-related behavior) of the unstable breathing. A combination of *in vivo* and *in situ* experimental strategies will be employed for this purpose. Specifically, we will determine when the respiratory phenotype occurs via plethysmography. Then we will stress the mice via exposure to the smell of urine from a predator to determine if the mutant mice have a propensity for a more disturbed respiratory pattern. We will then use the *in situ* working heart preparation to determine if the respiratory rhythm is abnormal. Our hypothesis is that a major component of the respiratory dysfunction is behavioral and thus will not occur in the reduced *in situ* preparation. Once the mechanisms underlying the respiratory dysfunction are clear, we could further test potential pharmacotherapeutic approaches (such as ampakines) to alleviate some features of this devastating problem.

Recently, using three-dimensional magnetic resonance imaging, Miller et al. (2007) detected a multiple intracranial abnormalities in PWS including ventriculomegaly (100% of individuals), decreased volume of brain tissue in the parietal-occipital lobe (50%), sylvian fissure polymicrogyria (60%), and incomplete insular closure (65%). The specific mechanisms underlying these neuropathological abnormalities and their correlation with the clinical phenotype remain to be elucidated. It will be interesting to examine human PWS brainstem with improved imaging techniques to see if the anatomical defects are similar to the mouse model (Pagliardini et al., 2005). This is part of an ongoing collaborative study between the Greer laboratory and the imaging center at the University of Alberta hospital.

7.3 A POTENTIAL PHARMACOTHERAPEUTIC APPROACH TO ALLEVIATE CENTRAL RESPIRATORY DEPRESSION: AMPAKINES

Positive airway pressure and methylxanthine therapy remain the mainstays of therapy in some indications of respiratory depression, specifically OSA and apnea of prematurity (Peña & García, 2006). There is a need for improved pharmacotherapeutic interventions to treat respiratory depression including weak and unstable endogenous respiratory drive (e.g. prematurity, sleep apnea, central respiration disorders with respiratory dysfunction), and exogenous (drug-induced) respiratory depression. Increased understanding of the neurochemical control of respiration will help identify a basis for advances.

In the final project, we focused our efforts into preclinical studies of ampakines to alleviate opioid-induced respiratory depression without affecting the analgesia. The rationale for developing this potential pharmacotherapeutic approach was based on the following. First, although various candidates including cholinesterase inhibitors such as physostigmine and 5-HT$_{4A}$ agonists have been proposed as antagonistics for opiate-induced respiratory depression (Tsujita et al., 2007; Snir-Mor et al., 1983; Manzke et al., 2003), clinical studies demonstrated that physostigmine (Bourke et al., 1984) and 5-HT$_{4A}$ agonists (Lötsch et al., 2005) did not alleviate opiate-induced respiratory depression. A recent study reported an unwarranted administration of cholinesterase inhibitors can impair genioglossus and diaphragm muscle function (Eikermann et al., 2007). Second, activation of AMPA receptors positively modulates respiratory drive and rhythmogenesis in several brain regions including the preBötC (Greer et al. 1991; Funk et al. 1993, 1995; Thoby-Brisson et al. 2005). Ampakines are a diverse group of small molecules that activate subsets of these receptors (Nagarajan et al., 2001). Thus, we hypothesized that accentuation of AMPAR-mediated conductances with ampakines will counter depressive actions of opioids acting within the preBötC.

First, we tested whether the ampakine CX546, the only one commercially available (distributed by Sigma), could enhance weak endogenous respiratory drive and counter opioid-induced respiratory depression. Respiratory frequency and amplitude were measured in the following rat models: perinatal *in vitro* brainstem-spinal cord and medullary slice, juvenile *in situ* working heart-brainstem preparation, and newborn and adult *in vivo*. We found that administration of CX546 stimulated weak baseline respiratory frequency in perinatal *in vitro* preparations but not baseline breathing in older animals. Furthermore, pharmacologic depression of respiratory frequency was countered at all ages studied by the administration of CX546 *in vitro*, *in situ*, and *in vivo*. Significantly, CX546 countered opioid-induced breathing depression in all preparations, without altering analgesia as assessed by measuring the time to foot withdrawal in response to a thermal stimulus. These studies suggest that ampakines may be useful in preventing or reversing opioid-induced respiratory depression and apnea of prematurity.

After our ampakine CX546 paper was published (Ren et al., 2006d), Ogier et al. (2007) extended the ampakine study to the *MeCP2*-deficient mouse, a model of RTT. They reported that Bdnf expression and respiratory function improved after chronic ampakine treatment in the *MeCP2*-deficient mouse, suggesting a potential pharmacological means for alleviating some features of this devastating syndrome.

Further work evaluating the efficacy of the various currently available members of the ampakine family as respiratory stimulants toward applicability in clinical settings became possible after collaboration was established between the Greer laboratory and Cortex Pharmaceuticals. CX717, one of the ampakine family that is metabolically stable and without significant side effects when administered to primate models and humans was further tested with a focus in the opioid-induced respiratory depression. We demonstrated that CX717 (see chapter VI) counters fentanyl-induced depression of respiratory frequency without suppressing analgesia. The effective dose of CX717 was in the range deemed safe based on clinical trials examining its efficacy for cognitive disorders. We concluded that CX717 is an agent that enhances the safety of using opiate drugs while preserving the analgesic effects. This advancement could significantly improve pain management in a variety of clinical settings. Phase IIa clinical trials are ongoing.

In this thesis, analyses at the single cell level with whole cell recordings were not performed with ampakines including CX546 and CX717. To explore the cellular mechanisms underlying the ampakine action on the opioid-caused respiratory depression or endogenous weak drive, different types of respiratory neurons could be studied in voltage- and current-clamp to observe the effects of ampakines on respiratory drive current, membrane potential and firing rates. Then the response to focal pressure injection of AMPA, in the presence of TTX to block synaptically mediated effects, will be compared pre- and post-bath application of ampakines. These may help understand the mechanisms underlying the primary conditioning drive via AMPA type glutamate receptors in key respiratory nuclei..

It would be interesting to test whether various members of the ampakine family could alleviate weak and irregular central respiratory drive in perinates. Experimental models to assess the ability of ampakines to stimulate weak respiratory drive include the following. 1) *In vivo* prenatal rats: Fetal rat pups are viable and have an unstable respiratory pattern if delivered one day premature (Greer et al., 1995). We will administer ampakines (i.p.) to determine, via plethysmography, if they are effective in stabilizing the respiratory rhythm *in vivo*. 2) Preterm lambs: Similar to preterm human infants, preterm lambs often suffer from poor respiratory drive and can succumb to respiratory insufficiency as a result of periods of apnea and hypoventilation. Dr. Richard Harding (Monash University) has an excellent lab model of apnea of prematurity (Davey et al., 1996). The aim of this collaborative study is to determine whether CX717 can stimulate breathing, improve blood gases (improved oxygenation, carbon dioxide levels and pH), reduce the incidence and duration of central apnea, and improve the stability of breathing, in prematurely born lambs. If successful, the study could indicate that these compounds may also be effective in stimulating breathing and reducing central apnea in preterm infants. 3) Genetic mouse models for central respiratory disorders including PWS (new strain of *necdin*-mutant mice generated by Dr. Rachel Wevrick), RTT (*MeCP2*-deficient mice), CCHS (*Phox2b*, by collaboration with Dr. Gallego's research team in Paris, see Dubreuil et al., 2008). We will use these genetic mouse models to test the hypothesis that ampakine administration (acutely and chronically) will alleviate the depression and irregularity of central respiratory drive in these devastating disorders. It will be important to test the more potent and longer-lasting ampakines provided by Cortex.

We could further extend the ampakine research to treatment of weak endogenous drive (e.g. OSA and CSA) in adults. SA is prevalent in subjects after age 60, occurring with more frequency and severity in those with congestive heart failure and neurological disorders (Launois, 2007). Central apneas are often observed in addition to obstructive and mixed events. Data from our assessment of ampakines to accentuate synaptic drive to XII motoneurons and preBötC neurons are clearly relevant to designing treatment strategies for apnea in adults. The Greer laboratory is currently establishing collaboration with Dr. Richard Horner (Univesity of Toronto) to develop that avenue of research. Further, the combination of *in vivo* plethysmography and *in situ* working heart preparations could help to dissect and understand the cause of respiratory dysfunctions in the elderly (i.e. defects in central respiratory control verse lung and cardiovascular pathologies).

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